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Physiological Importance of 1,25(OH)$_2$D$_3$ Membrane-Initiated and Nuclear Actions in Chickens as a Function of Growth, and Maturation in Male and Female Chickens

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PHYSIOLOGICAL IMPORTANCE OF $1,25(OH)_2D_3$ MEMBRANE-INITIATED AND NUCLEAR ACTIONS AS A FUNCTION OF GROWTH, AND MATURATION IN MALE AND FEMALE CHICKENS

by

Birgitta Larsson

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ABSTRACT

Physiological Importance of $1,25(OH)_2D_3$ Membrane-Initiated and Nuclear Actions in Chickens as a Function of Growth, and Maturation in Male and Female Chickens

by

Birgitta Larsson, Doctor of Philosophy

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It is well established that $1,25(OH)_2D_3$ is a major regulator of calcium homeostasis. The steroid exerts its effects on its target organs by two mechanisms, a slow mechanism mediated by nuclear vitamin D receptors (nVDR), and a rapid mechanism mediated by the $1,25(OH)_2D_3$ membrane-associated rapid response steroid binding protein ($1,25D_3$-MARRS bp). In this dissertation, the physiological relevance of membrane initiated steroid signaling was investigated by studying the correlation of age in male and female chickens with the magnitude of responses to $1,25(OH)_2D_3$ in duodena from 7-, 14-, 28-, and 58-week-old birds. Measurements of $1,25(OH)_2D_3$ (130 pM) responsiveness as a function of age showed an age-related decrease in intestinal Ca^{2+} transport for both male and female birds. Western analyses on isolated basal lateral membranes (BLM) indicated a decreased expression of $1,25D_3$-MARRS bp with increasing age in male chickens, while its expression increased with age in female birds. Saturation analyses of $[^3H]1,25(OH)_2D_3$ binding to BLM revealed an allosteric

interaction identified as cooperative binding for both male and female birds. The degree of positive cooperativity increased with increasing age. Both genders showed a significant increase in dissociation constant (K_d) with increasing age, indicating decreasing affinity. No age-related changes in the number of binding sites, B_{max}, were observed in male chickens, while female birds showed an increase in B_{max} with increasing age. Data obtained in parallel binding analyses of [^{3}H]1,25$(OH)_2D_3$ to nuclear fraction nVDR in contrast, indicated an absence of cooperative binding and an absence of significant changes in K_d or B_{max} with age for both male and female birds. Membrane-initiated signal transduction by 1,25$(OH)_2D_3$ was assessed by determination of protein kinase C (PKC) and A (PKA) activities. Both male and female birds showed an age-related decrease in stimulation of PKC activity in response to 1,25$(OH)_2D_3$, while PKA activity increased. In conclusion, this dissertation demonstrates that there is a decrease in 1,25$(OH)_2D_3$-induced intestinal calcium uptake as a function of age in duodena of both male and female chickens, which can be correlated to a decreased affinity for 1,25$(OH)_2D_3$, and a decreased PKC activity.

(139 pages)

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CHAPTER I

INTRODUCTION

Background

Historical introduction

The role of sunlight in the production of vitamin D was realized at the beginning of the industrial revolution. As cities became population centers, children were often constrained to play in sunless alleyways, and consequently developed a severe bone disease, rickets, which was first reported by the mid-seventeenth century. During the industrial revolution rickets increased dramatically, especially in Northern Europe and North America, and by the later part of the nineteenth century, autopsy studies made in Leiden (The Netherlands) reported that 90% of all children raised in this city had the disease (1).

An epidemiological survey which included clinical observations from a number of physicians throughout the British Empire and the Orient, reported that the disease was rare in children living in the cities in Japan, China and India. Children living in the British Islands on the other hand had a high incidence of the disease (1, 2). Based on these observations, Palm recommended systemic sunbathing to prevent the disease. But at the time, scientists and physicians could not accept such a simple prevention method for such a devastating bone disease. In 1919, Hudshinsky demonstrated that exposure to radiation from a mercury arc lamp could cure rickets (3). Two years later, Hess and Unger showed by x-ray examination that exposure to sunlight decreased rickets symptoms in children, thereby giving further proof for sunlight's curative powers (4).

On the coastline of the British Islands and along the coast of the Scandinavian countries it was common practice to use fish liver oils to prevent and cure rickets. Mellanby gave scientific proof for fish liver oil's antirachitic ability when he reported that he could cure the disease in rachitic dogs by adding cod-liver oil to their diet (5). At first it was thought that the antirachitic property possessed by fish liver oil was due to vitamin A, but McCollum and coworkers showed that when vitamin A activity was destroyed by heat and oxidation, cod-liver oil continued to have antirachitic activity (6). As a result it was concluded that there was a new fat-soluble vitamin that was called vitamin D. But it was still not clear whether the antirachitic factor in fish liver oil and the factor generated in the skin after sunlight exposure were the same substance. This was resolved when Powers and coworker found that radiation from a mercury arc lamp and ingestion of cod-liver oil had identical healing effects on the disease (7).

In many countries today, vitamin D is added to milk and a number of other foods, resulting in an almost complete elimination of rickets.

Calcium homeostasis

Calcium is the most abundant mineral in the body. Most of the calcium is bound to proteins and deposited in bone and teeth. Bone is the major store of the ion, containing more than 97 % of all calcium. The rest of the calcium is complexed with phosphate or bound to proteins, with about 0.1 % of the total calcium in the blood plasma and extracellular fluids. Extracellular calcium in the blood and the interstitial fluids modulate a number of vital cellular processes, including thrombosis, muscle and nerve excitability, and proper bone formation. In order to fulfill its function, the level of calcium in blood

plasma needs to be regulated to within very close limits, between 8.8-11.3 mg/dl in humans (8). Of this calcium, only half is free ionized calcium. It is the ionized form that is biologically active in the extracellular environment, and it is this form that is monitored by the systemic calcium homeostasis.

Three major hormones control calcium levels in blood and other extracellular fluids, namely parathyroid hormone (PTH), calcitonin and vitamin D.

Parathyroid hormone (PTH) is a single, 84 amino acid polypeptide chain that is produced in the parathyroid gland from a 115-residue pre-proparathyroid hormone (9), and is secreted in response to hypocalcemia (10). It is essential for maintenance of calcium homeostasis through direct actions on bone and kidney and direct and indirect actions on the gastrointestinal tract (Fig.1.1). PTH exerts its actions on bone to release calcium into the extracellular fluid by a mechanism not completely understood. In addition to its classical catabolic effect on bone, PTH has also been shown to have anabolic effects. Anabolic effects have been reported after intermittent administration of PTH to rats and humans, resulting in increased bone formation (11-16). Thus, it appears that prolonged exposure to high concentrations of PTH causes bone resorption, while short-term exposure induces anabolic effects.

In the kidney, PTH increases renal calcium reabsorption and decreases renal phosphate reabsorption (17-19). An additional action of PTH in the kidney is the stimulation of the synthesis of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which then acts on intestine to increase absorption of dietary calcium. The increased plasma calcium levels, in turn act by a negative feed-back to depress secretion of PTH (20). Besides having an indirect effect on intestine, direct actions have also been shown. PTH has been

shown to stimulate calcium absorption in both rat and chick intestine (21-24).

Calcitonin is a single-chain 32-amino acid polypeptide produced by the parafollicular or C-cells of the thyroid gland, and is secreted in response to elevation in circulating ionized calcium levels (25). Calcitonin counteracts hypercalcemia by enhancing calcium excretion in the kidney tubule cells (26) and inhibiting bone resorption (27) (Fig. 1.2). The bone resorption is inhibited by direct inhibition of osteoclast functions (27, 28), including cell motility and enzyme activity (29, 30). Calcitonin also prevents the efflux of calcium from bone by an osteoclast-independent mechanism (31). In the kidney calcitonin decreases tubular reabsorption of calcium and phosphate and inhibits actions of 25-hydroxyvitamin D_3-1-α–hydroxylase, thus inhibiting the formation of 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Very few studies have investigated the effects of calcitonin on intestinal calcium transport.

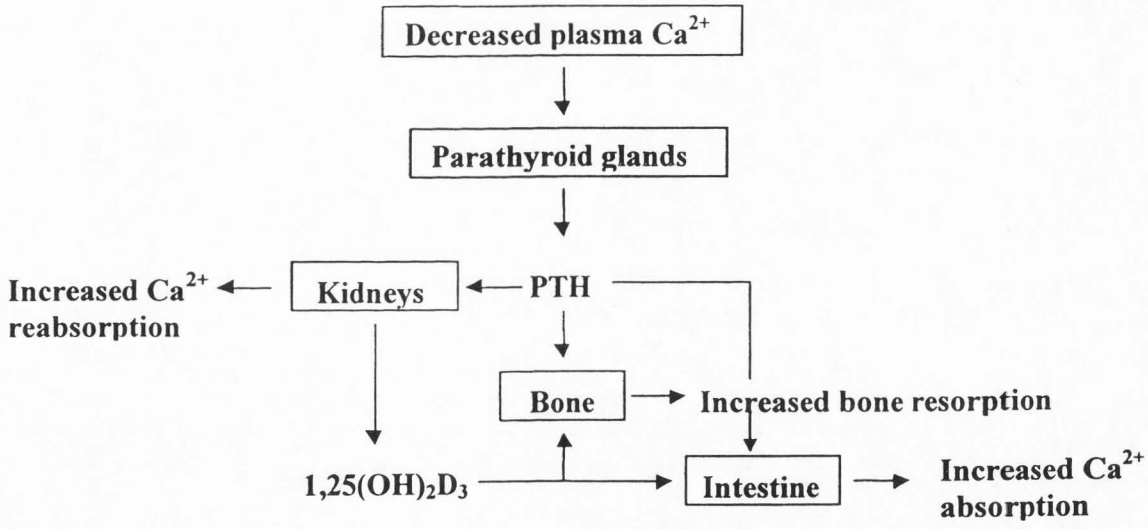

Fig. 1.1. Actions of PTH and $1,25(OH)_2D_3$ in calcium homeostasis.

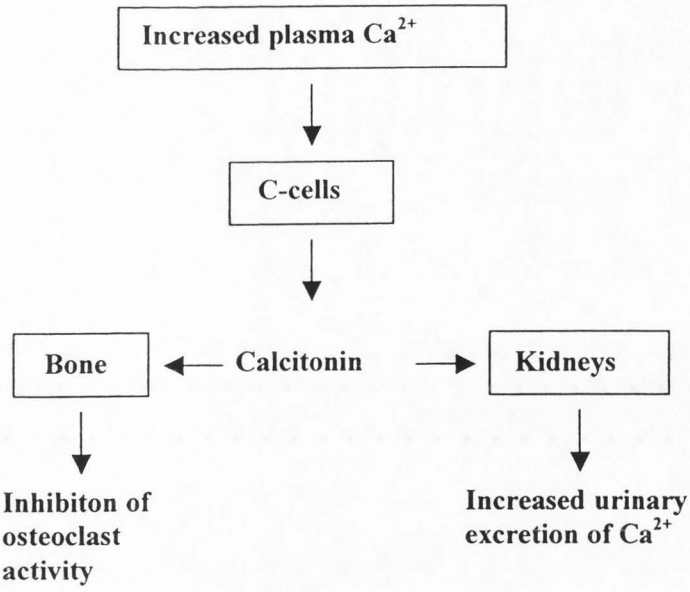

Fig. 1.2. Actions of calcitonin in calcium homeostasis.

Although the precise physiological role of calcitonin is not established, the ability of the hormone to decrease osteoclast activity is of therapeutic value. It has been used in the treatment of patients with high bone turnover and increased bone breakdown, including Paget's disease and postmenopausal osteoporosis (32-35).

Vitamin D is a principal factor required for the development and maintenance of bone as well as maintenance of normal calcium and phosphorous homeostasis. For vitamin D to affect calcium homeostasis, it has to be metabolized to its active form. Vitamin D is taken up from the diet or synthesized in the skin from 7-dehydrocholesterol in a reaction catalyzed by UV-light. The resulting pre-vitamin D_3 undergoes an internal isomerization to form vitamin D_3. Once vitamin D_3 is produced in the skin, it enters the circulation bound to the vitamin D-binding protein (DBP), and is transported to the liver.

In the liver, vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 ($25(OH)D_3$). The 25-hydroxyvitamin D_3 then proceeds to the kidney where it is further hydroxylated to form $1,25(OH)_2D_3$ or 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) (36). $1,25(OH)_2D_3$ is considered to be the primary biologically active form of vitamin D, responsible for maintaining serum calcium levels. Whether $24,25(OH)_2D_3$ is an active vitamin D metabolite is still a matter of debate, but increasing evidence shows that $24,25(OH)_2D_3$ also is of importance (37-41).

In the regulation of calcium homeostasis, $1.25(OH)_2D_3$ is considered to be a blood calcium-increasing hormone. In hypocalcemia, $1,25(OH)_2D_3$ increases intestinal absorption of calcium (42), decreases renal excretion of the ion (43), and releases bone calcium (44) (Fig. 1.1). As is the case for PTH, the mechanism by which $1,25(OH)_2D_3$ releases bone calcium is not known. Osteoblasts are thought to be the primary target cells for $1,25(OH)_2D_3$ because they have high levels of the nuclear vitamin D receptor (nVDR), which are absent in osteoclasts. Despite the lack of nVDR in differentiated osteoclasts, $1,25(OH)_2D_3$ has nevertheless been shown to have stimulatory actions on osteoclast-mediated bone resorption (45-47). Like PTH, $1,25(OH)_2D_3$ has, besides having a catabolic effect on bone, been shown to also exert an anabolic effect on the organ. The hormone is essential for bone formation, and it is well known that a lack of the steroid leads to rickets or osteomalacia, which can be corrected by administration of the vitamin (5, 48). On the other hand, similarly to PTH, administration of high doses for long periods leads to bone resorption (16, 49). Accordingly, $1,25(OH)_2D_3$ affects both the formation and resorption phases of bone metabolism.

In kidney there has been some controversy concerning the role of $1,25(OH)_2D_3$ in

renal calcium transport and the effects of the hormone are unclear. There are indications that $1,25(OH)_2D_3$ increases calcium transport. Vitamin D deficiency has been reported to decrease calcium reabsorption and to decrease the stimulatory effect of PTH on calcium reabsorption. Studies on distal tubule cells have shown that $1,25(OH)_2D_3$ enhances the actions of PTH by reducing the time needed for PTH to increase intracellular ^{45}Ca uptake. Furthermore, $1,25(OH)_2D_3$ has also been shown to increase PTH receptor messenger RNA and binding activity, suggesting one mechanism, whereby $1,25(OH)_2D_3$ may result in acceleration of PTH-dependent calcium entry (50, 51).

$1,25(OH)_3D_3$ also affects calcium homeostasis by inhibiting secretion and synthesis of PTH, thereby controlling its own production by a feed-back mechanism (20, 52, 53). In addition to inhibiting secretion and synthesis of PTH, $1,25(OH)_2D_3$ can decrease its own production by suppressing 25-hydroxyvitamin D_3-1-α–hydroxylase and stimulating the renal 25-hydroxyvitamin D_3 24-hydroxylase enzyme that hydroxylates $25(OH)D_3$ and $1,25(OH)_2D_3$ to $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, respectively (54, 55).

In the intestine $1,25(OH)_2D_3$ is known to increase calcium uptake by two mechanisms, a rapid membrane initiated one, and a slow genomic one.

Intestinal calcium transport

Calcium uptake over the small intestine occurs by two independent processes. The first is a non-saturable, possibly paracellular route, which is concentration dependent, essentially independent of nutritional and physiological regulation and takes place all along the small intestine (56). The non-saturable component of calcium transport is driven by differences in calcium concentration in the intestinal lumen and the body fluids.

The calcium ion concentration in body fluids is approximately 1.2-1.5 mM. This means that if the luminal calcium concentration is greater than 1.5 mM the ion will move down a concentration gradient from the intestinal lumen to the basal lateral body fluids (57).

The second process that takes place largely in the proximal intestine, the duodenum and upper jejunum, is a transcellular route. This process is dependent on nutritional and physiological regulation via vitamin D. For calcium to be transported by this route it needs to first be taken up by the enterocyte at the brush border (luminal/apical side), transported across the enterocyte and finally extruded at the basal lateral (blood) side (Fig. 1.3). The interior of the cell is negatively charged in comparison to the exterior. And the fact that the calcium concentration of the intestinal contents may be in the mM range, while the concentration of calcium within the cell is in the μM range, results in an electrochemical gradient from the lumen to the interior of the cell. Therefore the movement of calcium across the apical membrane does not require energy, and cell transporters or channels mediate the uptake at the apical membrane (57, 58). The channels responsible for the uptake over the apical membrane are L-type voltage directed calcium channels (L-type VDCC) (59), calcium transport protein 1 (CaT1) also called epithelial calcium channel 2 (ECAC2) (60, 61) and cyclic nucleotide-gated channels (CNG) (62). An alternative or perhaps related uptake route at the apical membrane is vesicle mediated, where calcium is taken in to the cells by endocytosis (63-66). Once inside the enterocyte, calcium needs to be transported through the cell to the basal lateral side, where it is extruded. This process needs energy because concentrations of calcium in extracellular fluids and blood are higher than those within the cell, and because the cell is electronegative relative to the extracellular fluid compartment. Two transport

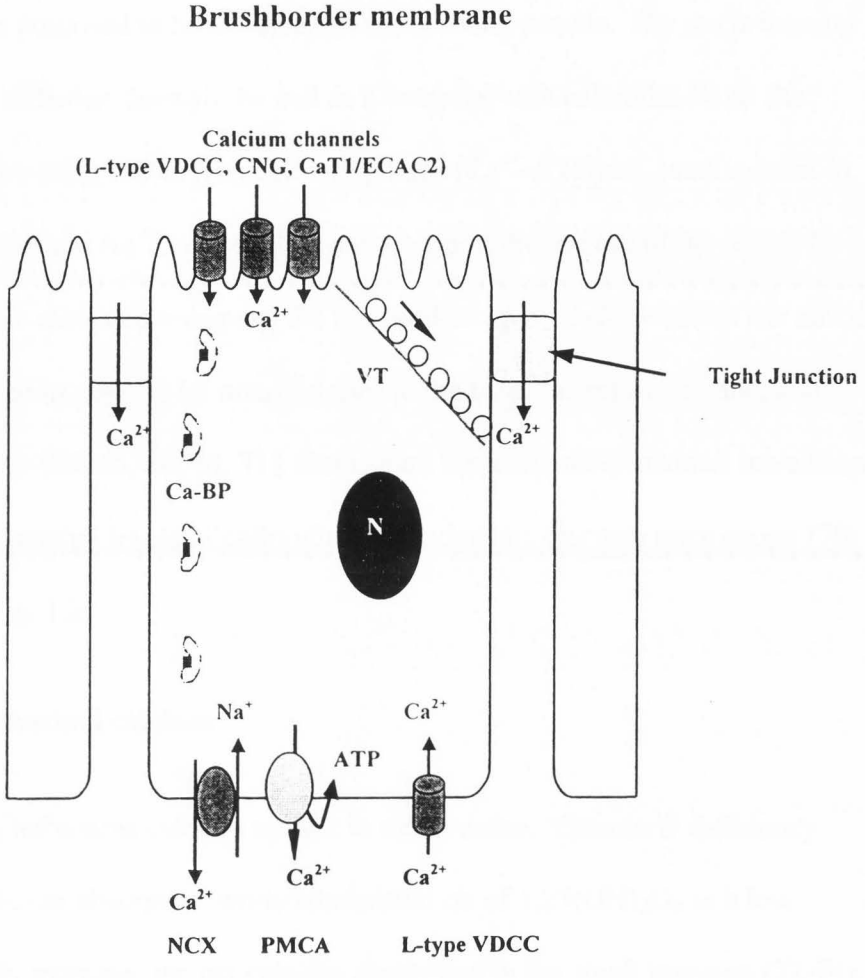

Fig. 1.3. Calcium transport over the intestine. VT stands for vesicular transport, Ca-BP for calcium binding protein and N for nucleus. L-type VDCC stands for L-type voltage directed calcium channels, CNG stands for cyclic nucleotide gated channels and CaT1/ECAC2 stands for calcium transporter protein 1/epithelial calcium channel 2. NCX is an abbreviation for Na^{+}/Ca^{2+}- exchanger and PMCA stands for plasma membrane Ca^{2+}-ATPase.

mechanisms have been proposed, one mediated by calcium binding proteins and one vesicle mediated (57, 63-66). In the calcium binding protein mediated mechanism, calbindin D has been proposed to be the calcium transporting protein. The ion is thought to be transported by diffusion through the cell as a complex with calbindin D. At the basal lateral end of the cell, ATP-driven calcium pumps (Ca^{2+}-ATPases, predominant in birds and mammalians) and Na^{+}/Ca^{2+}- exchangers transport the ion out of the cell (64, 67-69). For the vesicle mediated transport, the ion is taken up by endocytosis at the apical membrane, transported in vesicles by microtubules to the basal lateral membrane, and extruded by exocytosis (63, 65, 66, 70, 71). Both small vesicles and lysosomes have been shown to contain substantial levels of calbindin D as judged by electron microscopy (70). For a summary see Fig. 1.3.

$1,25(OH)_2D_3$ and intestinal calcium transport

$1,25(OH)_2D_3$ influences calcium uptake in the intestine. Vitamin D deficiency reduces duodenal calcium absorption while administration of $1,25(OH)_2D_3$ or a low calcium diet markedly increases the net calcium absorption in the small intestine (72-74). The cellular mechanisms by which $1,25(OH)_2D_3$ enhances calcium absorption are not clear. Several reports have demonstrated the importance of new protein synthesis in calcium transport, and $1,25(OH)_2D_3$ influences the expression of calbindin D and Ca^{2+}-ATPase thereby affecting calcium absorption (72, 75-77). However, Bikle *et al.* (78) found $1,25(OH)_2D_3$-stimulated transport in vitamin D deficient chicks to be independent of de novo mRNA and protein synthesis. Moreover, perfusion experiments in chick have shown that, following infusion of $1,25(OH)_2D_3$ into the celiac artery of the perfused

intestinal loops, there is a rapid increase in calcium transport from the intestinal lumen into the extracellular fluid space independent of the synthesis of new protein (66, 70, 71). Evidence has been obtained indicating that these processes are associated with the modulation of L-type VDCC (79, 80). Furthermore, increases in intracellular second messengers cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and protein kinase C (PKC) have been postulated to mediate the activation of the channels or other steps in the transport process (79, 81-85).

Based on these observations one may conclude that $1,25(OH)_2D_3$ affects the intestinal calcium absorption by two mechanisms, a slow process dependent on protein synthesis, and a rapid process independent of de novo synthesis of proteins.

Slow and rapid actions of $1,25(OH)_2D_3$

In the slow mechanism, which is in the range of min, hours, or days, $1,25(OH)_2D_3$ mediates its effects by binding to nuclear vitamin D receptors (nVDR). These receptors are macromolecules with molecular weights ranging from ~48,000 for humans to ~60,000 for avian species, and possessing at least two unique binding domains, a carboxy-terminal vitamin D binding domain and an amino-terminal DNA-binding region (44, 72, 86). In order to exert its effect $1,25(OH)_2D_3$ needs to bind to nVDR which is most concentrated within the cell nucleus, but has also been found in the cytoplasm. It was thought for a long time that $1,25(OH)_2D_3$ is translocated over the cell membrane by diffusion, but recent data indicate that a membrane associated $1,25(OH)_2D_3$ rapid response steroid binding protein ($1,25D_3$-MARRS bp) may be responsible for the transport of $1,25(OH)_2D_3$ over the cell membrane and into the nucleus (85). Once inside

the cell $1,25(OH)_2D_3$ binds to either monomers of the nVDR or to heterodimeric complexes composed of nVDR and retinoid X receptor (RXR) accessory proteins (nVDR-RXR). The nVDR-RXR complex then binds to regulatory vitamin D response elements (VDRE) on the DNA and affects transcription. In vitro studies have shown that $1,25(OH)_2D_3$ is not necessary for the binding of nVDR-RXR to the VDRE (87). The role of $1,25(OH)_2D_3$ in the transcription process is not clear, but it is thought that $1,25(OH)_2D_3$ initiates the movement of nVDR into the nucleus and, furthermore, it has been shown that binding of $1,25(OH)_2D_3$ to nVDR enhances the binding of nVDR-RXR heterodimers to the VDRE, possibly by increasing the binding affinity (87, 88). After binding of the receptor complex to the VDRE, evidence indicates that a phosphorylation of the nVDR is important in the activation of the hormone-receptor complex. This phosphorylation leads to modified interactions with other transcription factors bound by the gene promoter region (89-91). As a result gene transcription can be either induced or repressed by signal transduction pathways involving phosphorylation/dephosphorylation, resulting in changes in the protein expression (Fig. 1.4). Today nVDRs have been identified in at least 35 target tissues and over 50 genes are known to be regulated by $1,25(OH)_2D_3$ (92).

The rapid pathway is thought to be mediated by $1,25(OH)_2D_3$ interacting with a putative protein receptor located in the cell membrane. The putative receptor $1,25D_3$-MARRS bp, responsible for the translocation of $1,25(OH)_2D_3$ over the cell membrane, are thought to be the same molecule. Specific binding of $1,25(OH)_2D_3$ to basal lateral membranes has been reported in chicks and anti sera has been raised to the N-terminus of the protein (85, 93). The binding of $1,25(OH)_2D_3$ to its putative receptor results in an

increased intracellular protein kinase activity, opening of calcium channels and an overall increase in intestinal calcium transport (94, 95). These effects are observed within seconds to minutes after $1,25(OH)_2D_3$ administration compared to hours and days required for the slow effects. The slow and the rapid pathways are summarized in Fig. 1.4.

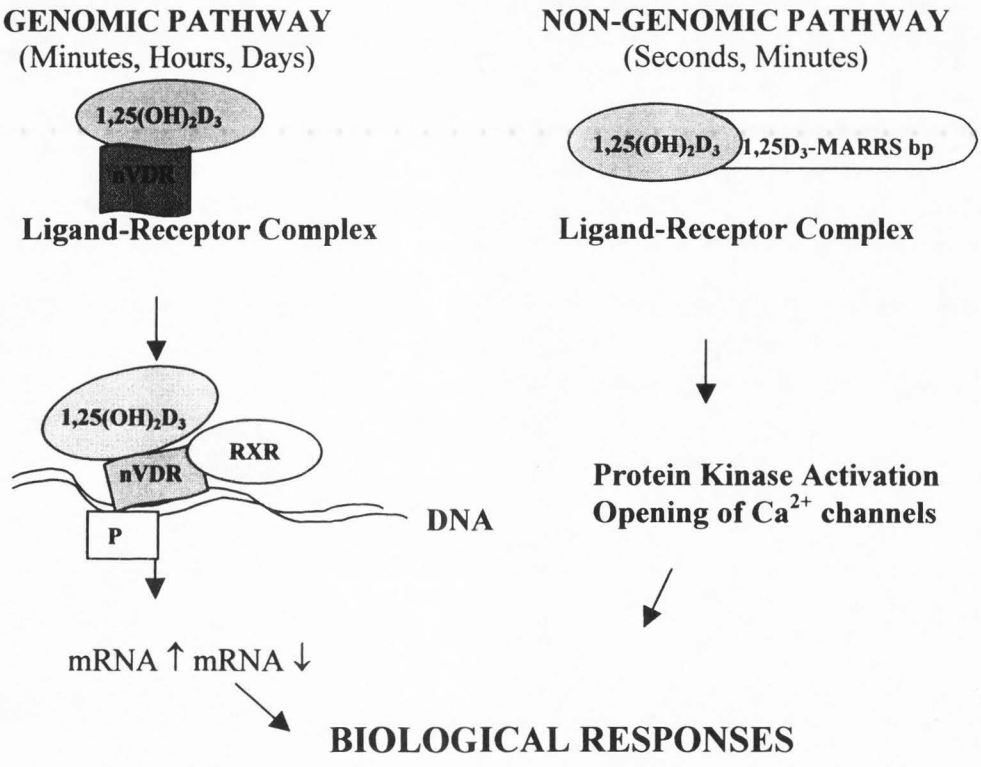

Fig. 1.4. Pathways for generation of biological responses by $1,25(OH)_2D_3$. RXR stands for retinoid X receptor, nVDR stands for nuclear vitamin D receptor, $1,25D_3$-MARRS bp stands for $1,25(OH)_2D_3$ membrane associated rapid response steroid binding protein and P stands for phosphate.

Hypothesis and specific aims

A hormonally active metabolite of vitamin D, $1,25(OH)_2D_3$, is known to regulate calcium and phosphate metabolism through a genomic mechanism mediated by nuclear vitamin D receptors (nVDR). The seco-steroid hormone is also capable of activating the transport pathway by interacting with $1,25D_3$-MARRS bp and initiating signal transduction pathways.

The hypothesis underlying this research is that the non-nuclear receptor is physiologically important in maintaining calcium homeostasis. We sought to test this hypothesis in male and female chickens as a function of age, gender, and maturation. While animals are growing, large amounts of dietary calcium are required to build bones in both sexes. As the animal reaches adulthood, roosters would have less need of rapidly stimulated intestinal transport while hens are expected to continue to require large amounts of calcium to produce eggshells. Thus, the rapid effect of $1,25(OH)_2D_3$ on intestinal calcium transport, mediated through the $1,25D_3$-MARRS bp, is expected to decrease in roosters, while hens may be expected to have a sustained responsiveness to $1,25(OH)_2D_3$. The hypothesis was shown to be correct for male birds. Female birds on the other hand did not sustain responsiveness to $1,25(OH)_2D_3$ with increasing age, thus disagreeing with the hypothesis.

The specific aims of the proposed research were to:

1. Follow $1,25(OH)_2D_3$-induced intestinal calcium uptake in male or female chickens as a function of growth and maturation.
2. Characterize the $1,25D_3$-MARRS bp with respect to binding parameters (number of

unoccupied receptor binding sites, B_{max}, and dissociation constant, K_d) and expression in basal lateral membranes isolated from male or female chickens of varying ages.

3. Characterize the nuclear vitamin D receptor, nVDR, in crude nuclear fractions with respect to binding parameters (B_{max} and K_d) in intestinal epithelium from male or female chickens of varying ages.
4. Follow $1,25(OH)_2D_3$ induced signal transduction via protein kinase C (PKC) and protein kinase A (PKA) in enterocytes from male or female chickens of varying ages.

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CHAPTER II

EFFECT OF GROWTH AND MATURATION ON MEMBRANE-INITIATED ACTIONS OF 1,25-DIHYDROXYVITAMIN D_3. I. CALCIUM TRANSPORT, RECEPTOR KINETICS, AND SIGNAL TRANSDUCTION IN INTESTINE OF MALE CHICKENS[1,2]

Abstract

To study the physiological relevance of membrane initiated steroid signaling, we investigated the effects of age in male chickens on the magnitude of response to $1,25(OH)_2D_3$ in duodena from 7-, 14-, 28-, and 58-week-old birds. Measurements of $1,25(OH)_2D_3$ (130 pM) responsiveness as a function of age, showed a decreased intestinal Ca^{2+} transport. Western analyses on isolated basal lateral membranes (BLM) indicated a decreased expression of the membrane associated rapid response steroid binding protein ($1,25D_3$-MARRS bp) with increasing age. Saturation analyses of $[^3H]1,25(OH)_2D_3$ binding to BLM, revealed an allosteric interaction identified as positive cooperative binding. A significant increase in the dissociation constant, K_d, was observed with increasing age, indicating a decreasing affinity. Determinations of the number of binding sites yielded B_{max} = 190-250 fmol/mg protein during growth and maturation, while in adulthood (58 wks), saturable binding was no longer observed. Data obtained in parallel binding analyses of $[^3H]1,25(OH)_2D_3$ to nuclear fraction nVDR in contrast,

[1]Coauthered by Birgitta Larsson and Ilka Nemere.
[2]Submitted for publication in Endocrinology.

indicated an absence of cooperative binding and an absence of significant changes in K_d or B_{max} with age. Membrane-initiated signal transduction by $1,25(OH)_2D_3$ was assessed by determination of protein kinase C (PKC) and A (PKA) activities. Stimulation of PKC activity in response to $1,25(OH)_2D_3$ decreased with age, while age did nor affect steroid-stimulated PKA activities. In conclusion, our experiments demonstrated that there is a decrease in rapid induction of intestinal calcium uptake by exogenous $1,25(OH)_2D_3$ as a function of age in duodena of male chickens, which can be associated with a decreased affinity for $1,25(OH)_2D_3$, a reduced expression of $1,25D_3$-MARRS bp, and a decreased PKC activity.

Introduction

Aging is associated with a declining efficiency of intestinal calcium absorption in humans and other vertebrates (1-3). It has been suggested that this impaired efficiency in calcium absorption is related to the inability of elderly persons to maintain a normal calcium balance, increasing the risk of bone loss and osteoporotic fracture (4). 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], an active hormonal form of vitamin D, is considered to be the principal regulator of calcium transport. Thus, changes in the vitamin D endocrine system with age may be implicated in the pathogenesis of calcium malabsorption.

Aging may affect the vitamin D endocrine system in different ways. A low dietary intake (5), inadequate exposure to sunlight (6), and a progressive decline in renal function with age, resulting in a decreased 25-hydroxyvitamin D 1α–hydroxylase activity (7, 8), may lead to decreased levels of $1,25(OH)_2D_3$. The decreased levels of $1,25(OH)_2D_3$ in

turn may cause a reduced calcium uptake. Further, the capacity of $1.25(OH)_2D_3$ to stimulate calcium absorption also declines with age (9, 10), but the biochemical mechanisms responsible for this decrease in intestinal function are not fully understood.

$1,25(OH)_2D_3$ effects target organs by two mechanisms. The most studied is the classical nuclear receptor mediated pathway, where $1,25(OH)_2D_3$ binds to the nuclear vitamin D receptor (nVDR) and initiates a cascade of events resulting in genome activation and transcription (11). In intestine, this transcriptional activity is required to provide a biochemically competent enterocyte containing all necessary components of the calcium transport pathway (12). In addition, $1,25(OH)_2D_3$ affects a variety of rapid biological responses in intestine as well as in other classic and non-classic target tissues. These rapid responses occur too quickly to be explained by genome activation (13, 14), and include changes in intracellular protein kinase C (PKC) and A (PKA) activities (15, 16). In the biochemically competent enterocyte, the rapid response results in enhancement of calcium transport (12). It has been proposed that $1,25(OH)_2D_3$ mediates its rapid effects by interacting with a *m*embrane *a*ssociated *r*apid *r*esponse *s*teroid binding protein ($1,25D_3$-MARRS bp) located in the plasmalemma (17), rather than a nuclear receptor (18). Specific binding of $1,25(OH)_2D_3$ to basal lateral membranes has been reported in chicks and anti-sera has been raised to the N-terminus of the putative receptor protein (18, 19). The binding of $1,25(OH)_2D_3$ to its putative receptor results in an over all increase in intestinal calcium transport, observed within seconds to minutes after $1,25(OH)_2D_3$ administration compared to hours and days required for the classical pathway.

Both the classical nVDR- and membrane-initiated pathways may be involved in

decreasing intestinal $1,25(OH)_2D_3$ responsiveness as a function of age (20-26). Even though evidence exists for the involvement of both pathways, they have not been evaluated in the same animals. Moreover, in the studies on the membrane initiated pathways in rats (24-26), changes in signal transduction activation were not related to receptor binding, nor membrane-initiated changes in transport. Thus, further knowledge of the mechanisms resulting in senescence related changes in $1,25(OH)_2D_3$ responsiveness are important to fully understand the pathogenesis of calcium malabsorption.

The present study explored age-related changes in intestinal calcium transport due to changes in responsiveness to $1,25(OH)_2D_3$. The rapid activation of calcium transport in the male avian intestine by $1,25(OH)_2D_3$ was compared to the steroid-activated intracellular second messenger systems, PKC and PKA. Further, characteristics of the nVDR and $1,25D_3$-MARRS bp receptors were studied by looking at changes in receptor affinity and maximal binding capacity as a function of growth and maturation.

Materials and methods

Materials

White leghorn cockerels were from Merrill Poultry, Poul, ID. Vitamin D-supplemented diet (1.0 % calcium, 1.0 % phosphorus) was from Nutrena Feeds (Murray, UT), chloropent from Fort Dodge Laboratories, (Fort Dodge, IA), $^{45}CaCl_2$ and $[^3H]1,25(OH)_2D_3$ were from NEN Life Science Products Inc (Boston, MA). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Bradford, MA), kits for PKC and PKA determination were from Life Technologies-GIBCO (Waverly, MA),

Bradford dye was from Bio-Rad (Hercules, CA) and [γ-^{32}P]ATP was purchased from NEN. All other chemicals were of highest grade available and obtained from Sigma Chemicals Co, St Louis, MO.

Animals

White leghorn cockerels were obtained on the day of hatch and raised on a vitamin D-supplemented diet prior to experiment. Animals of ages 7, 14, 28 and 58 weeks were studied. Growth phases were represented by 7 and 14 weeks, while 28 and 58 weeks were representative of adulthood. On the day of the experiments, chickens were anesthetized with 0.3 ml chlorohydrate sodium pentabarbital/100g B.W.

Perfusion studies

Perfusion studies were performed as described earlier (27). In brief, luminal perfusion was performed with Gey´s balanced salt solution (GBSS), lacking bicarbonate and containing 1 μCi $^{45}CaCl_2$/ml. Each perfusion experiment was divided into three periods (Fig. 2.1). During the first period (10 min), vascular perfusion with aerated GBSS-control medium (containing 0.005 % ethanol) was conducted to allow the system to reach a steady state. During the second period (10 min) samples of venous effluent are collected for assessment of basal ^{45}Ca transport. Throughout the third period the preparation was exposed to either GBSS-control medium (pefusion of 7, 14, 28, and 58 week old birds n = 4, 3, 5, and 5, respectively) or 130 pM $1,25(OH)_2D_3$ in GBSS (pefusion of 7-, 14-, 28-, and 58-week-old birds n = 6, 3, 4, and 3, respectively) for 40 min and radioactivity assessed in the collected fractions. The transport during the third period was normalized to the corresponding average basal transport rate according to the

following formula:

$$\frac{cpm_t}{\sum_{t=-10}^{T=0} cpm/5} = T_{(0\text{-}40)}$$

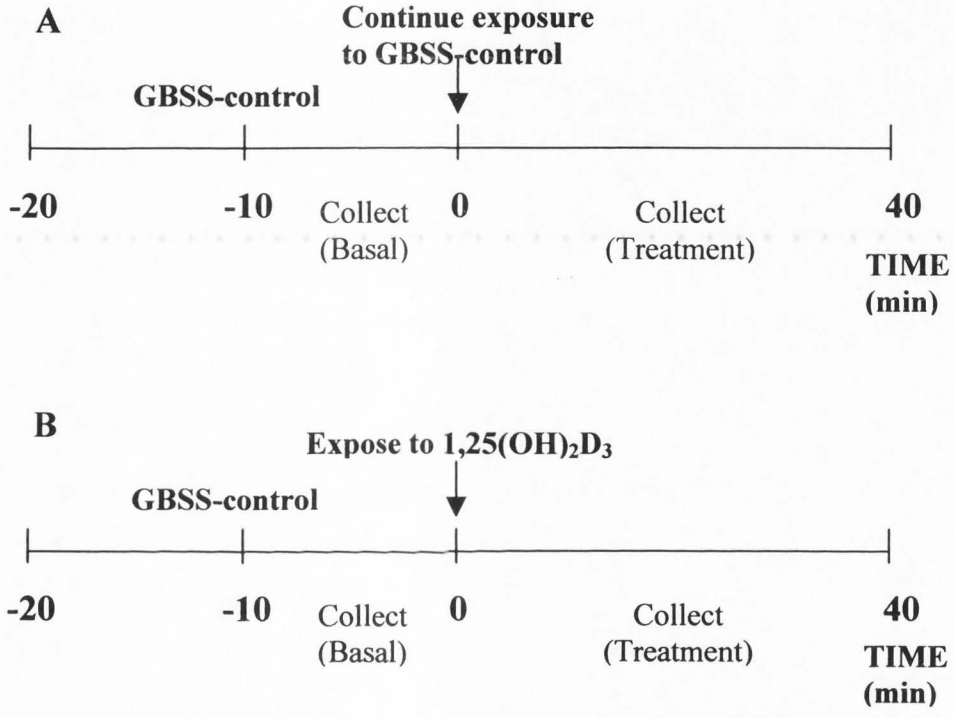

Fig. 2.1. Experimental design of perfusion studies on duodena from male chickens. Duodenal loops of male chickens aged 7, 14, 28 or 58 weeks were vascularly perfused with either GBSS-control media or 130 pM $1,25(OH)_2D_3$ in GBSS. Fig. 2.1A shows the experimental design for perfusion with GBSS-control medium and Fig. 2.1B represents perfusion with 130 pM $1,25(OH)_2D_3$. In both control and $1,25(OH)_2D_3$ treatment the duodena were vascularly perfused with GBSS-control medium between –20 to 0 min. Venous effluent was collected at time –10 to 0 min for assessment of basal ^{45}Ca transport. At time 0 min the vascular perfusion with GBSS-control medium was either continued for 40 min (A) or the treatment with GBSS-control medium was stopped and the animals were instead vascularly perfused with $1,25(OH)_2D_3$ in GBSS (B). Aliquots of venous effluent were collected between time 0 to 40 min (treatment period) and ^{45}Ca assessed.

Preparation of crude nuclei and basal lateral membranes

Subcellular fractions were prepared (n = 3 for each age group) as reported earlier (18, 28). Intestinal epithelium was disrupted in 40 ml homogenization medium (250 mM sucrose, 5 mM histidine-imidazole, 2 mM EGTA, pH 7.0) with a Dounce homogenizer and a teflon pestle. The epithelium was fractioned by differential centrifugation, resulting in a crude nuclear fraction, and an intracellular organelle fraction containing the basal lateral membranes (BLM). Intracellular organelles were separated from BLM by centrifugation in Percoll. Eighteen fractions were collected from the gradient (52 drops each). Fractions 16-18 containing BLM (18, 28) were pooled and the Percoll removed by ultra centrifugation (18). Crude nuclei and BLM were stored at –20 °C until analyses.

Saturation analysis

BLM fractions were adjusted to 50 μg of protein per tube and incubated in TED buffer (10 mM Tris, 2 mM EDTA and 1 mM dithiothreitol, pH 7.4) overnight (0 °C) with 0.5, 1.0, 2.0, or 4.0 nM [^{3}H]1,25$(OH)_2D_3$ in the absence (total binding) or in the presence of a 200-fold molar excess of unlabeled 1,25$(OH)_2D_3$ (nonspecific binding). Each sample was assayed in triplicate for total and nonspecific binding. Nuclear fractions from each group were incubated in the same way as BLMs, but with [^{3}H]1,25$(OH)_2D_3$ concentrations of 0.25, 0.5, 1.0 or 2.0 nM.

After incubation overnight, bound and free ligands were separated by either perchloric acid precipitation for binding to BLM (18) or by a hydroxylapatite (HAP) assay for binding to nuclear fractions (29).

For BLM fractions, perchloric acid and carrier protein (γ-globulin) were added to

each tube, the mixture was incubated on ice for 20 min, and the precipitated protein pelleted by centrifugation. The pellets were solubilized in guanidinium/HCl, decanted into vials, liquid scintillation cocktail added, and the amount of [^{3}H]1,25(OH)$_2$D$_3$ assessed.

While the perchloric acid method is suitable for hydrophobic membrane proteins (1,25D$_3$-MARRS bp), it fails to detect the nVDR (18). Saturation analysis of [^{3}H]1,25(OH)$_2$D$_3$ binding was therefore assessed in crude preparations of nuclei by the use of a HAP assay as reported elsewhere (29). Briefly, after incubation with different concentrations of [^{3}H]1,25(OH)$_2$D$_3$ over night (see above), 200 µl HAP and 800 µl TED containing 0.5 % Triton X-100 was added to each tube, and the solutions were mixed. The HAP-bound receptor was pelleted by centrifugation, washed twice with 800 µl TED containing 1.5 % Triton X-100, treated with 1 ml ethanol to extract ligand, and centrifuged. The resulting supernatant was transferred to glass scintillation vials, the ethanol evaporated, and the amount [^{3}H]1,25(OH)$_2$D$_3$ measured after addition of fluor.

SDS-PAGE and Western analysis

SDS-PAGE and Western blot analysis were used to determine immunoreactive levels of 1,25D$_3$-MARRS bp. Proteins (15 µg/well; [19]) were separated on an 8 % sodium dodecylsulfate (SDS) polyacrylamide gel with a 5 % stacking gel. After separation on SDS-PAGE, proteins were transferred to a PVDF membrane by the use of a Trans-Blot SD Semi-dry transfer cell (Bio Rad) and Western analyses were performed according to the Millipore protocol as follows. In order to avoid non-specific binding, the membrane was soaked for 1 h at 37 °C in blocking solution, 0.5 % non-fat dry milk in

phosphate buffered saline (PBS; 0.9 % NaCl, 10 mM Na_2HPO_4, pH 7.4), followed by washing 3 × 5 min with washing solution, 0.1 % (w/v) BSA in Tris buffered saline (TBS; 0.9 % NaCl in 20 mM Tris-HCl pH 7.4), and incubation with primary antibody Ab099 (rabbit anti-1,25D_3-MARRS bp N-terminal peptide; dilution 1/5000 in 1 % BSA, 0.05 % Tween-20 in TBS) (19) over night at 4 °C. After 3 additional 3 × 5 min washes with washing solution, the membrane was incubated with secondary antibody (alkaline phosphatase conjugated goat anti rabbit IgG), in 1 % BSA, 0.05 % Tween-20 in TBS for 2 h at room temperature, and then washed as before. Immunoreactive bands were visualized with the chromogens BCIP/NBT, and relative amounts of 1,25D_3-MARRS bp were quantitated by using densitometry and computer software.

Determination of PKC and PKA and protein activities

Intestinal epithelial cells from two male chicken duodena per preparation experiment were isolated by citrate chelation as reported elsewhere (30). After removing the duodenum, chilling it for 15 min in saline and excising the pancreas, the duodenum was everted, rinsed in saline, and the intestinal epithelial cells isolated by chelation. The isolated cells were combined, centrifuged at 500 × g, 4 °C for 5 min and the resulting pellet resuspended in 20 ml GBSS-BSA (119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM Na_2HPO_4, 1.03 mM $MgCl_2$, 0.28 mM $MgSO_4$, 0.9 mM $CaCl_2$, pH 7.4 + 0.125% BSA). Cells were treated at room temperature with either ethanol or 130 pM 1,25$(OH)_2D_3$ at time zero. For PKC, cells were incubated for 5 min, and for PKA the incubation lasted for 7 min (31). After incubation the cells were centrifuged at 1000 × g, 4 °C for 10 min, the supernatant decanted, and the pellet stored at –20 °C until used.

PKC and PKA activities were analyzed by the use of commercially available assay systems. The analyses and activity calculations were performed according to instructions packaged with the kits (Appendix B). Enzyme activity was extracted by homogenizing cell pellets in the appropriate extraction buffer followed by incubation on ice for 30 min and centrifugation. For PKC activity 10 μg of supernatant fraction protein were incubated at room temperature for 20 min in the presence of PKC activator or inhibitor. [γ-^{32}P]ATP (20-25 μCi/ml) was added to each tube, the mixture was incubated for 5 min at 30 °C, and 25 μl of the mixture spotted onto a phosphocellulose disc. The discs were washed, transferred to scintillation vials, incorporated ^{32}P assessed, and specific PKC activity calculated. For PKA activity, proteins (5 μg) from the supernatant fractions were incubated at room temperature for 20 min in the presence of activator or inhibitor only, in the presence of both activator and inhibitor and in the absence of both. [γ-^{32}P]ATP (20-25 μCi/ml) was added to each tube, the mixture was incubated for 5 min at 30 °C, and 20 μl of the mixture spotted onto a phosphocellulose disc. The discs were washed, transferred into scintillation vials, ^{32}P assessed and PKA activity calculated.

Proteins were determined using the Bradford dye according to manufacturer's instructions (Appendix B).

Statistics and data analysis

Data available from perfusion studies (I. Nemere, unpublished observations) were analyzed by Student's t-test for unpaired observations within each age group, between treated and controls. For trend analysis between age groups, linear regression was performed with values of T/Av basal at 40 min (see below).

The specific binding of [^{3}H]1,25(OH)$_2$D$_3$ to receptor was calculated and plotted against the corresponding concentration of [^{3}H]1,25(OH)$_2$D$_3$ The data were analyzed by non-linear regression analysis by fitting either to a three parameter sigmoid equation or a hyperbolic function.

The sigmoid equation was as follows:

$$Y = \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

where Y is specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (fmol/mg protein), x is the concentration of 1,25(OH)$_2$D$_3$ (nM) in the incubation mixture, a is the maximum specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (B_{max}), b is the minimum specifically bound [^{3}H]1,25(OH)$_2$D$_3$ and x_0 is the concentration of 1,25(OH)$_2$D$_3$ in the incubation mixture at half maximum bound [^{3}H]1,25(OH)$_2$D$_3$ (K_d).

For the hyperbolic function, the equation was as follows:

$$Y = \frac{a * x}{b + x}$$

where Y is specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (fmol/mg protein), x is the incubation concentration of 1,25(OH)$_2$D$_3$ (nM) in the incubation mixture, a is the maximum specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (B_{max}) and b is the concentration of 1,25(OH)$_2$D$_3$ in the incubation mixture at half maximum bound [^{3}H]1,25(OH)$_2$D$_3$ (K_d).

Data from Hill analysis, perfusion studies and SDS-PAGE/Western blots were analyzed by linear regression. The coefficient of determination (R^2) and the adjusted coefficient of determination ($_{adj}R^2$) were used as measures of how well the regression model describes the data. A one-way analysis of variance (ANOVA) with F-statistics was

used to gauge the contribution of the independent variable to predict the dependent variable. The significance level was set at $P<0.05$, and data are presented as mean ± SEM. When comparing B_{max}, K_d, and Hill coefficients between different age groups an unpaired Student's t-test was used. When variables were in more than one comparison a sequentially rejective Bonferroni test (32) was used. In the PKC and PKA measurements, and in comparison of changes in basal intestinal calcium uptake statistical comparisons were performed using one-way ANOVA followed by Student's Newman-Keuls posthoc test when comparing a factor with more than two levels. The tests used were two-tailed, and the significance level was set at $P<0.05$. Data are presented as mean ± SEM.

Results

Effect of age on intestinal calcium transport

Male chickens of ages 7, 14, 28, and 58 weeks were used in the studies, and had average weights (mean ± SEM) of 0.56 ± 0.02, 0.96 ± 0.03, 1.28 ± 0.03, and 2.53 ± 0.05 kg, respectively. Average basal calcium transport (T = -10 to 0 min) was determined to be 1.2 ± 0.5, 0.6 ± 0.2, 1.6 ± 0.4, and 0.9 ± 0.3 μg ^{45}Ca in the venous effluent/min for 7, 14, 28, and 58 weeks old birds, respectively. No significant changes in basal calcium transport with age were observed.

Vascular perfusion with 130 pM $1,25(OH)_2D_3$ enhanced intestinal calcium transport in all ages studies (Fig. 2.2) As shown in Fig.2.2A-D treatment with $1,25(OH)_2D_3$ showed a linear increase in intestinal calcium transport for all ages. The difference between control medium-treated and $1,25(OH)_2D_3$-treated duodena from 7, 14,

28, and 58 week old animals became significant at T = 4, T = 20, T = 4, and T = 6 min, respectively. At 40 min, 7, 14, 28, and 58 week old birds attained treatment/average basal ^{45}Ca values in venous effluent of 2.6 ± 0.4, 1.9 ± 0.4, 1.8 ± 0.4, and 1.5 ± 0.2, respectively, while the controls were 0.9 ± 0.1, 1.0 ± 0.1, 1.0 ± 0.1, and 0.9 ± 0.1, respectively (Fig 2.2). Accordingly, stimulated calcium transport in duodena from 7, 14, 28, and 58 week birds went from 290 % of controls to 190 %, 180 %, and 170 % of controls, respectively, at T = 40 min.

Linear regression analysis showed a significant ($P<0.05$) age-dependent decreasing trend in stimulated calcium transport when comparing ^{45}Ca in venous effluent expressed as (cpm at T = 40 min) / (average basal cpm) for the different ages ($P<0.05$, F = 4.8 DF = 15, $R^2 = 0.20$, and $_{adj}R^2 = 0.25$) (Fig. 2.3).

Saturation analysis of $1,25(OH)_2D_3$ binding to nVDR

Specific binding activity of nVDR, as a function of increasing concentrations of ligand was determined in nuclear fractions prepared from intestinal mucosa of 7-, 14-, 28-, and 58-week-old male birds (Fig. 2.4). The data were fitted to a hyperbolic function ($P<0.05$ for all ages, 7 weeks; F = 85, DF = 11, $R^2 = 0.89$ and $_{adj}R^2 = 0.88$, 14 weeks; F = 62, DF = 9, $R^2 = 0.89$ and $_{adj}R^2 = 0.87$, 28 weeks; F = 40, DF = 11, $R^2 = 0.80$ and $_{adj}R^2 = 0.78$, and 58 weeks; F = 110, DF =7, $R^2 = 0.95$ and $_{adj}R^2 = 0.94$). Maximal binding capacity, (B_{max}) for 7, 14, 28, and 58 weeks old birds was calculated to be 31 ± 3.9, 36 ± 5.2, 31 ± 5.1, and 23 ± 2.6 fmol/mg protein, respectively. In the same age groups, specific binding became half-saturable at a K_d of 0.4 ± 0.1, 0.4 ± 0.2, 0.3 ± 0.2, and 0.3 ± 0.1 nM, respectively. No significant differences in B_{max} or K_d with increasing age were observed.

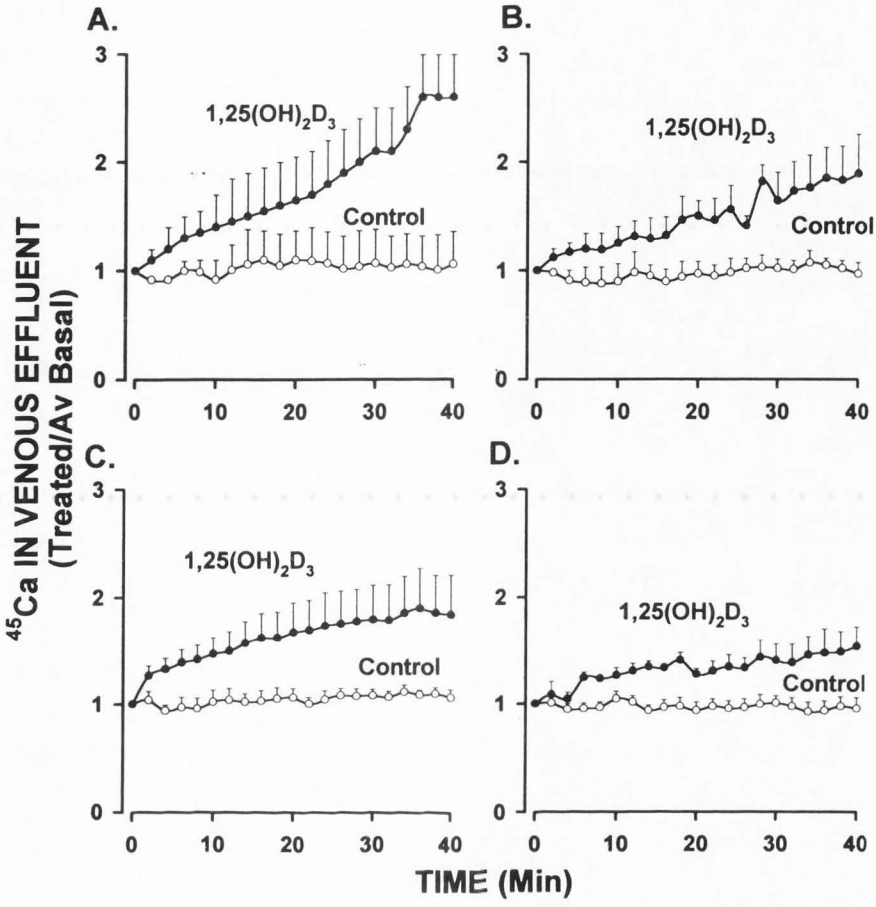

Fig. 2.2. Effects of age on the rapid stimulation of intestinal calcium transport by $1,25(OH)_2D_3$ in male duodena. Isolated duodena of male chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were vascularly perfused with the vehicle ethanol (0.005%, final concentration) during the basal phase, and again in the treated phase for controls (open symbols), or with 130 pM $1,25(OH)_2D_3$ during the treated phase (closed symbols). Values represent ^{45}Ca in the venous effluent during the treatment period normalized to the corresponding average basal transport determined at T= -10 to 0 min.

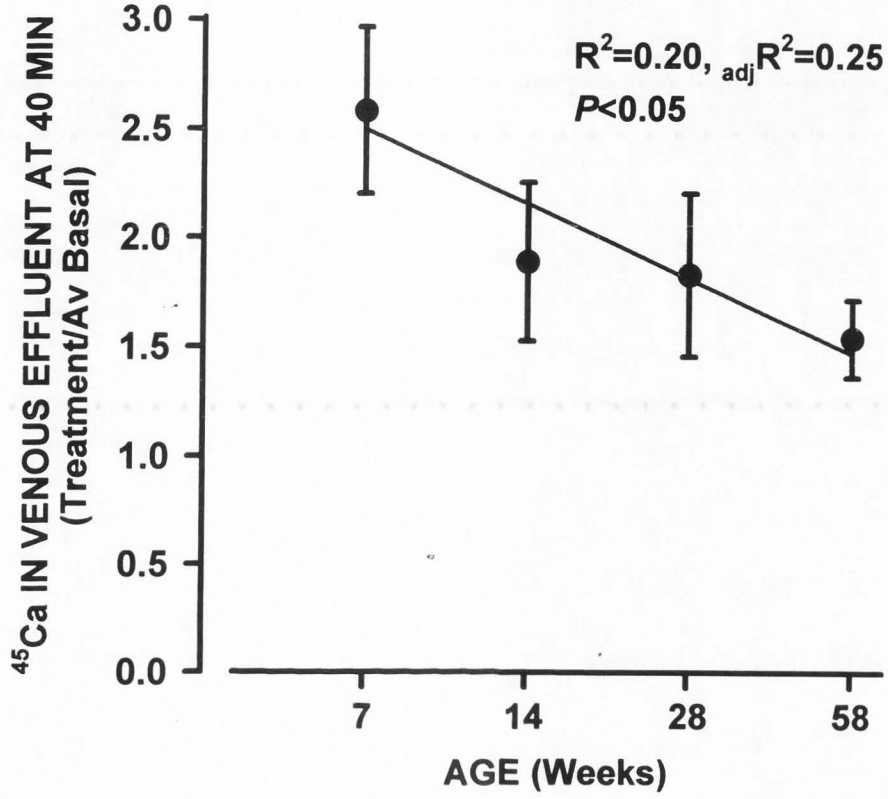

Fig. 2.3. Changes in ^{45}Ca in venous effluent with age in male chickens. Isolated duodena of male chickens of ages 7, 14, 28, and 58 weeks were vascularly perfused with the vehicle ethanol (0.005%, final concentration) during the basal phase, and in the treated phase with 130 pM $1,25(OH)_2D_3$. Values represent ^{45}Ca in the venous effluent at T = 40 min normalized to the corresponding average basal transport determined at T= -10 to 0 min. A linear regression analysis of the data revealed a significant (P<0.05) decrease in $1,25(OH)_2D_3$ induced intestinal calcium uptake with increasing age. R^2 is the coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. Data are presented as mean ± SEM (n = 3-6).

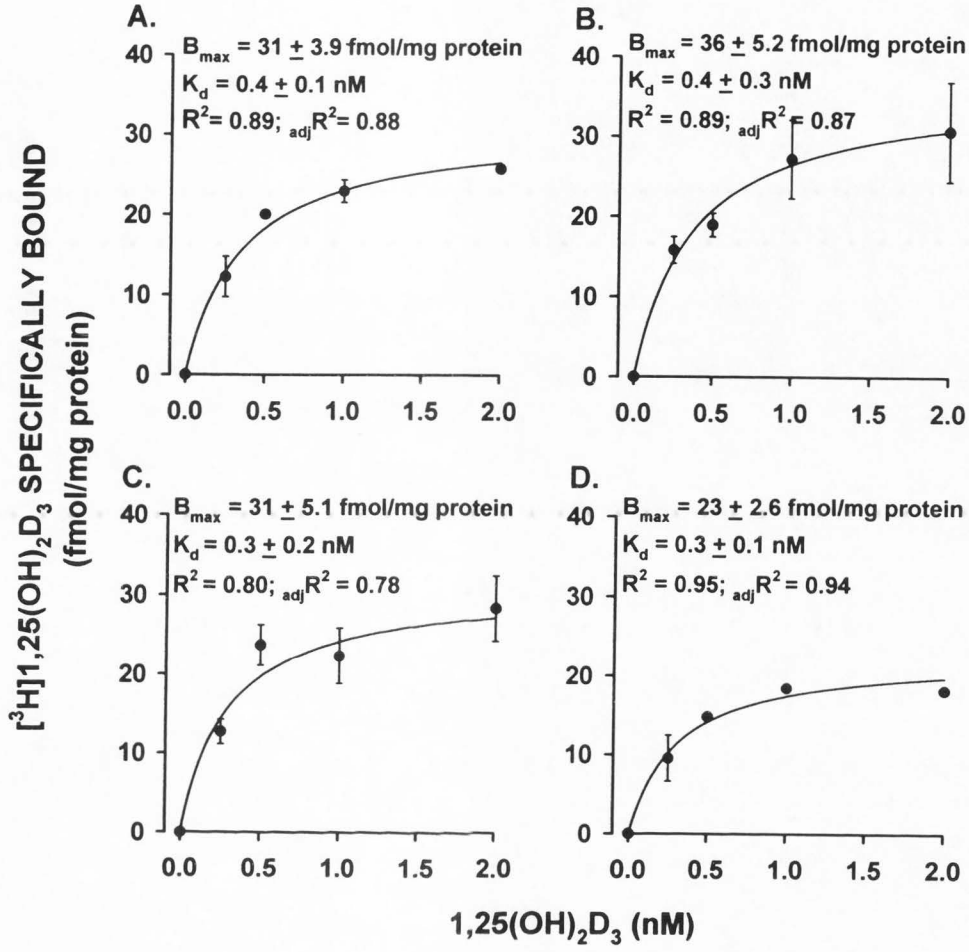

Fig. 2.4. Saturation analysis of $1,25(OH)_2D_3$ binding to nVDR in crude nuclear fractions from male chickens. Crude nuclear fractions enriched in nVDR isolated from male chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were incubated with 0.25, 0.5, 1.0, or 2.0 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or in the presence of 200-fold molar excess of unlabeled steroid. Bound radioactivity was assessed by the hydroxyl apatite assay. Data were fitted to a three parameter hyperbolic function and tested by nonlinear regression. R^2, coefficient of determination and, $_{adj}R^2$, the adjusted coefficient of determination. No significant differences in maximum binding capacity, B_{max}, or dissociation constant, K_d, with increasing age were observed when determined by Student's t-test. $P<0.05$ for significance and data are presented as mean ± SEM for three independent preparations in each age group.

Data described in Fig. 2.4A-D were recalculated for Hill analysis, which yielded curves that could be described by a linear regression ($P<0.05$ for all ages, 7 weeks; F = 20, DF = 11, $R^2 = 0.66$ and $_{adj}R^2 = 0.63$, 14 weeks; F = 5.4, DF = 7, $R^2 = 0.48$ and $_{adj}R^2 = 0.39$, 28 weeks; F = 9.2 DF = 9, $R^2 = 0.53$ and $_{adj}R^2 = 0.48$, and 58 weeks; F = 36, DF =7, $R^2 = 0.86$ and $_{adj}R^2 = 0.83$). The resultant apparent Hill coefficients (n_{app}) were 1.0 ± 0.2, 0.8 ± 0.3, 1.0 ± 0.3, and 1.5 ± 0.3 for 7, 14, 28, and 58 weeks birds, respectively (Fig. 2.5A-D). No significant difference in n_{app} was observed for the different ages. Taken together, both the hyperbolic distribution and the slope in the Hill analysis indicate absence of cooperative binding. With regards to maximal binding and affinity of nVDR for $1,25(OH)_2D_3$ no changes were observed with increasing age.

Saturation analysis of $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS bp and Western analysis

Fig. 2.6A-D shows the specific binding activity as a function of increasing concentrations of ligand in BLM fractions prepared from intestinal mucosa of 7-, 14-, 28-, and 58-week-old male birds. Curve fitting analyses of the data resulted in a better fit to a sigmoid function compared to a hyperbolic function. ($P<0.05$ for all ages, 7 weeks; F = 20.0, DF = 24, $R^2 = 0.65$ and $_{adj}R^2 = 0.61$, 14 weeks; F = 100, DF = 14, $R^2 = 0.94$ and $_{adj}R^2 = 0.93$, 28 weeks; F = 72, DF = 14, $R^2 = 0.92$ and $_{adj}R^2 = 0.91$, and 58 weeks; F = 80, DF =14, $R^2 = 0.93$ and $_{adj}R^2 = 0.92$) indicating presence of allosteric binding. Values of K_d were calculated to be 0.6 ± 0.1, 1.0 ± 0.1, 1.0 ± 0.1, and 2.7 ± 0.8 nM for 7-, 14-, 28-, and 58-week-old birds, respectively. A significant increase in K_d was observed between 7- and 14-week-old birds. This decreased affinity was sustained through the subsequent ages tested. Values of B_{max} for 7-, 14-, 28-, and 58-week-old birds were

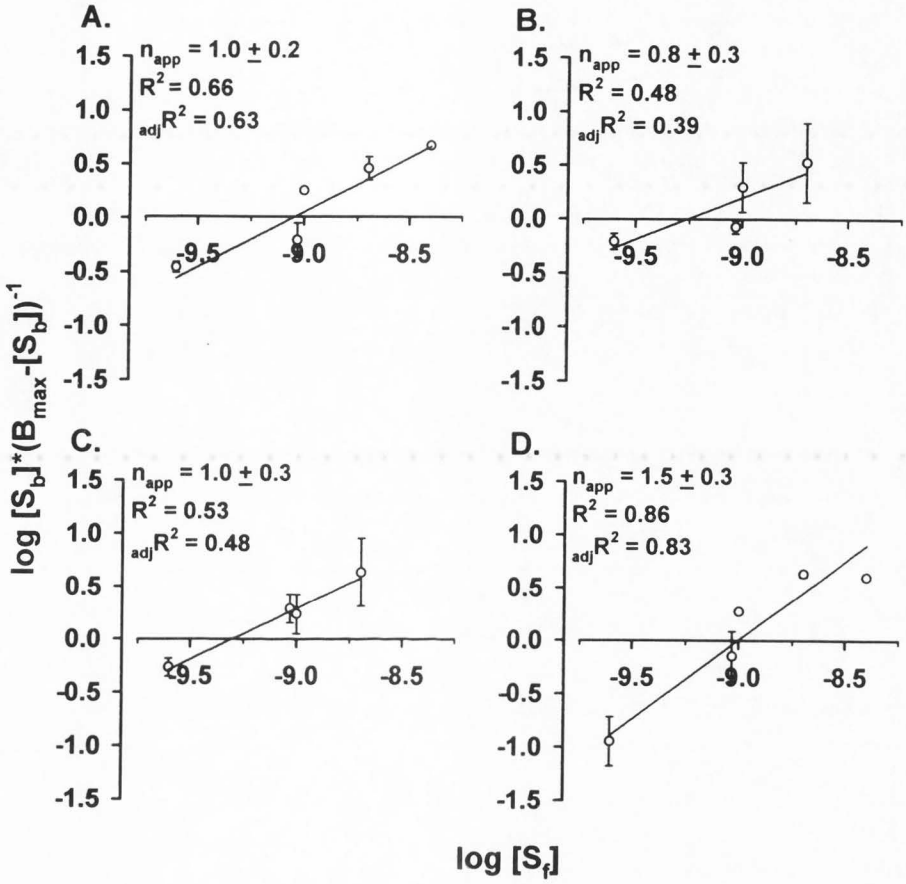

Fig. 2.5. Hill analysis of $1,25(OH)_2D_3$ binding to nVDR in crude nuclear fractions from male chickens. Data presented in Fig. 2.4 A-D were recalculated for Hill analysis. (**A**) 7-, (**B**) 14-, (**C**) 28-, and (**D**) 58-week-old birds. $[S_b]$, specifically bound fraction of the administered total free ($[S_f]$) $[^3H]1,25(OH)_2D_3$, R^2, coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. No significant differences in apparent Hill coefficient (n_{app}) were observed with increasing age when compared by Student's t-test. An n_{app} equal to 1 shows the presence of noncooperative binding for $1,25(OH)_2D_3$ to nVDR. Data are presented as mean ± SEM (n = 3 per tested $[^3H]1,25(OH)_2D_3$ concentration) and $P<0.05$ for significance.

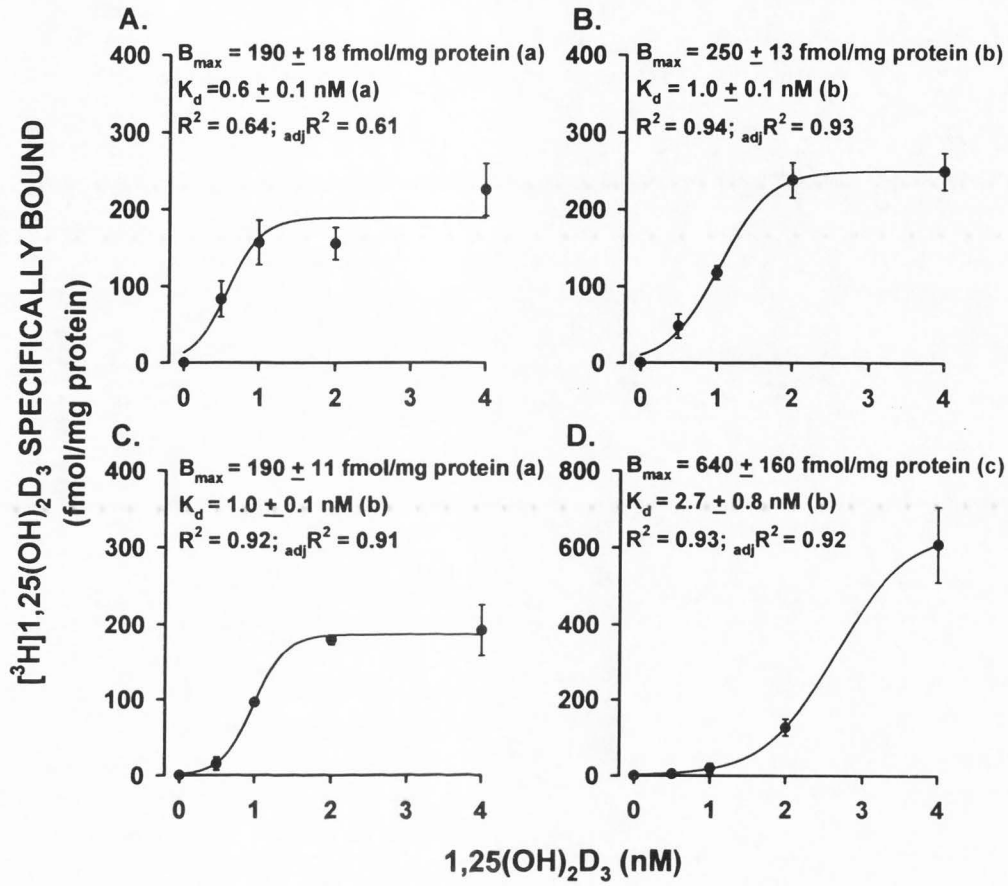

Fig. 2.6. Saturation analysis of $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS bp in BLM from male chickens. Basal lateral membranes (BLM) isolated from male chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were incubated with 0.05, 1.0, 2.0, or 4.0 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or presence of a 200-fold molar excess of unlabeled steroid. Bound radioactivity was assessed by precipitation with perchloric acid in the presence of carrier bovine γ-globulin. Data were fitted to a three parameter sigmoid function and tested by nonlinear regression. R^2 is the coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. Significant differences in maximum binding capacity, B_{max}, and dissociation constant, K_d, with increasing age were determined by Student's t-test. Small letters in brackets indicate significant differences ($P<0.05$) between ages. Data are presented as mean ± SEM (n = 3 independent preparations in each age group).

calculated to be 190 ± 18, 250 ± 13, 190 ± 11, and 640 ± 160 fmol/mg protein, respectively, although the last value cannot actually be used as a measure of binding sites, since saturation was not achieved at the concentrations tested (Fig. 2.6D). A statistically significant difference was observed between B_{max} values for 7-, 14-, and 58-week-old birds. The 28-week-old birds showed a significant difference in B_{max} compared to 14- and 58-week-old birds, while there was no significantly difference between 28- and 7-week-old birds (Fig. 2.6A-D).

A recalculation of the data in Fig. 2.6A-D for Hill analysis resulted in curves that were described by linear regression ($P<0.05$ for all ages, 7 weeks; F = 8.1, DF = 12, R^2 = 0.24 and $_{adj}R^2$ = 0.37, 14 weeks; F = 53, DF = 9, R^2 = 0.87 and $_{adj}R^2$ = 0.85, 28 weeks; F = 23, DF = 9, R^2 = 0.74 and $_{adj}R^2$ = 0.71, and 58 weeks; F = 113, DF =9, R^2 = 0.93 and $_{adj}R^2$ = 0.93). The Hill analysis yielded Hill coefficients greater than 1 for all ages indicating the presence of positively cooperative binding of $1,25(OH)_2D_3$ to $1,25D_3$-MARRS bp. The n_{app} were calculated as 1.2 ± 0.4, 2.1 ± 0.3, 2.5 ± 0.5, and 3.2 ± 0.3 for 7-, 14-, 28-, and 58-week-old birds, respectively (Fig. 2.7A-D). Statistical comparison of n_{app} between the ages revealed a significant difference between 7- and 58-week-old birds and 14- and 58-week-old birds. No significant differences were found when comparing 14- to 28-week-old birds and 28- to 58-week-old birds.

For each age group, three independent BLM preparations were subjected to Western blotting with Ab 099 for analyses of $1,25D_3$-MARRS bp levels. Multiple blots were subjected to densitometric analyses for quantitation. Linear regression ($P<0.05$, DF = 39, F = 4.6, R^2 = 0.11 and $_{adj}R^2$ = 0.09) analysis of the data obtained revealed a decreasing trend in expression of $1,25D_3$-MARRS bp with increasing age (Fig. 2.8).

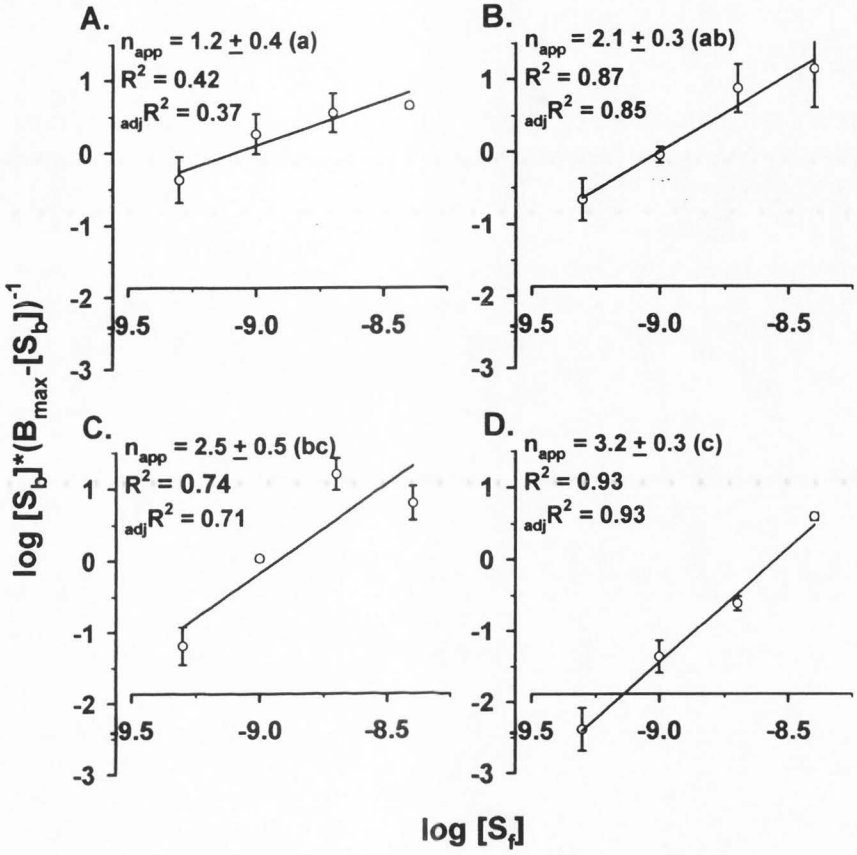

Fig. 2.7. Hill analysis of $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS bp in BLM from male chickens. Data presented in Fig. 2.6 A-D were recalculated for Hill analysis. (**A**) 7-, (**B**) 14-, (**C**) 28-, and (**D**) 58-week-old birds. [S_b], specifically bound fraction of the administered total free ([S_f]) [^{3}H]$1,25(OH)_2D_3$, R^2, coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. The apparent Hill coefficients (n_{app}) were larger than 1 in all age groups, indicating positive cooperative binding for $1,25(OH)_2D_3$ to $1,25D_3$-MARRS bp. Small letters in brackets indicate significant (P<0.05) differences determined by Student's t-test. Data are presented as mean ± SEM.

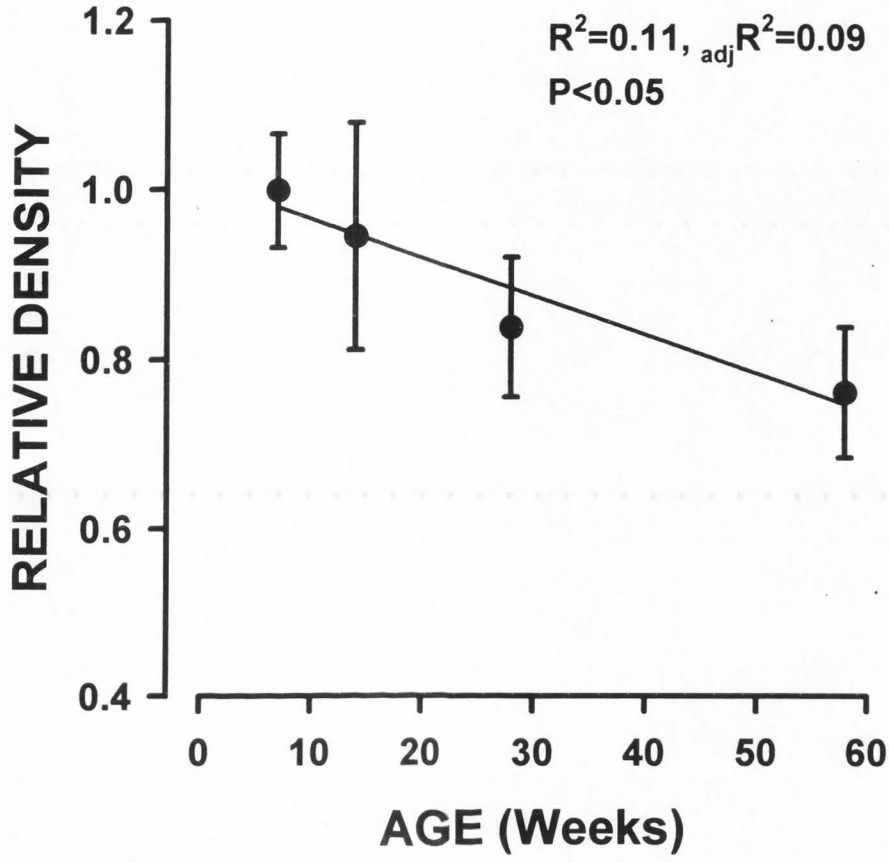

Fig. 2.8. Expression of 1,25D_3-MARRS bp in BLM from male chickens. The expression of 1,25D_3-MARRS bp in basal lateral membranes (BLM) isolated from duodenal epithelium of male chickens ages 7, 14, 28, and 58 weeks, was studied by separation of proteins on 8% SDS-PAGE followed by Western blot analysis. For Western analysis, Ab 099 (rabbit anti-1,25D_3-MARRS bp N-terminal peptide) was used as primary antibody and alkaline phosphatase conjugated goat anti rabbit IgG as secondary antibody. Immunoreactive bands were visualized with the chromogens BCIP/NBT and relative amounts of 1,25D_3-MARRS bp were quantitated by using densitometry and computer software. A linear regression analysis of the data revealed a significant (P<0.05) decrease in 1,25D_3-MARRS bp expression with increasing age. R^2 is the coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. Data are presented as mean ± SEM (n = 8-12) from 4 separate blots, each containing samples from all age groups.

Thus, the results demonstrate a decrease in both expression of $1,25D_3$-MARRS bp and affinity of the receptor with increasing age, and further, a presence of positive cooperativity in the binding of $1,25(OH)_2D_3$ to $1,25D_3$-MARRS bp.

Effects of age on $1,25(OH)_2D_3$ induced PKC and PKA activities

Despite the significant changes in $1,25D_3$-MARRS bp affinity and levels, it was recognized that differences in intestinal responsiveness to steroid might be due to pleiotropic changes at the cellular level between growing and mature animals. Thus, the effects of 130 pM $1,25(OH)_2D_3$ on PKC and PKA activities in isolated intestinal cells from male chickens of different ages were tested.

Figure 2.9 illustrates the results of PKC activity determinations in parallel incubations of control and hormone treated cells from 7-, 14-, 28-, and 58-week-old birds. Extracts from all age groups were assayed concomitantly in order to validate comparisons of basal (control) levels. As shown in Fig. 2.9 the PKC activity was affected by age in both vehicle treated (control) and $1,25(OH)_2D_3$ treated cells. PKC activity in vehicle treated enterocytes exhibited an age related decrease between 7 and 14 weeks yielding values of 140 ± 9.9, 53 ± 5.9, 43 ± 8.4, and 20 ± 2.9 pmol/min/mg protein ($n = 3$-14) for 7-, 14-, 28-, and 58-week-old birds, respectively, for the tested ages. Upon comparison of treatment and corresponding controls within each age group an increase in activity was observed after treatment with hormone in isolated cells from 7-week-old animals, while enterocytes from 58-week-old birds showed a decreased PKC activity after hormone treatment (Fig. 2.9). No changes in PKC activity were observed within each age group when comparing hormone-treated cells with vehicle-treated cells from

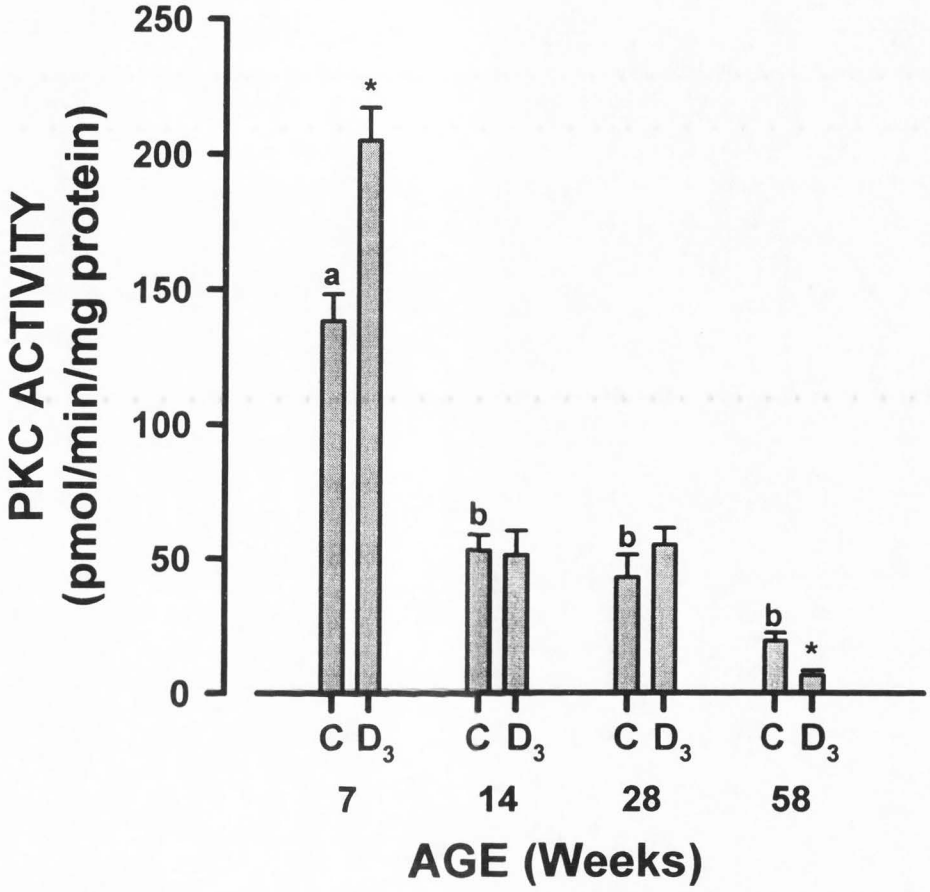

Fig. 2.9. PKC activity in male chicken enterocytes. Intestinal epithelial cells from 7-, 14-, 28-, and 58-week-old male chickens were isolated and treated with either 130 pM $1,25(OH)_2D_3$ (D_3) or ethanol (C) for 5 min. The cells were collected by centrifugation, extracted, and the supernatant fractions analyzed for protein kinase C (PKC) activity. Significant differences between D_3 and C within each age group (*) and differences between C for the different age groups (small letters) were obtained by a one-way ANOVA followed by Student's Newman-Keuls post-hoc test ($P<0.05$). Data are presented as mean ± SEM (n = 4-14).

14 and 28 week old animals (Fig. 2.9). The results of analyses of PKA activity in vehicle- and $1,25(OH)_2D_3$-treated cells from 7-, 14-, 28-, and 58-week-old male chickens are shown in Fig. 2.10. The activities in vehicle-treated cells from 7-, 14-, 28-, and 58-week-old birds were 26 ± 2.5, 31 ± 8.5, 29 ± 0.3, and 21 ± 8.2 % activated PKA/mg protein (n = 3-6), respectively, and for $1,25(OH)_2D_3$-treated cells in the same age groups, 55 ± 6.3, 50 ± 4.7, 58 ± 12, and 58 ± 2.2 % activated PKA/mg protein (n = 3-5), respectively (Fig. 2.10). No effect of age was observed in PKA activity when comparing vehicle-treated cells. $1,25(OH)_2D_3$ however increased PKA activity in 7- and 58-week-old birds.

Figure 2.11A and B show PKC and PKA activities, respectively, in $1,25(OH)_2D_3$-treated cells as percent of controls. The changes in PKC activity were calculated as 160 ± 14, 100 ± 21, 140 ± 17, and 34 ± 8.7 % (n = 4-14), respectively, for 7-, 14-, 28-, and 58-week-old birds. When comparing $1,25(OH)_2D_3$ induced PKC activities between ages a significant decrease in PKC activity was observed between 28- and 58-week-old birds (Fig. 2.11A). Steroid mediated changes in PKA activity, expressed as percent of controls, were calculated to be 200 ± 21, 160 ± 15, 200 ± 42, and 270 ± 10 % (n = 3-6), respectively, for 7-, 14-, 28-, and 58-week-old birds (Fig. 2.11B). When comparing $1,25(OH)_2D_3$-stimulated PKA activity between ages 14 week old birds showed a significant lower induction than 28 week old birds. No differences in induced PKA activities were observed when comparing 7-, 28-, and 58-week-old birds.

Discussion

In this work evidence was obtained indicating that $1,25(OH)_2D_3$-induced

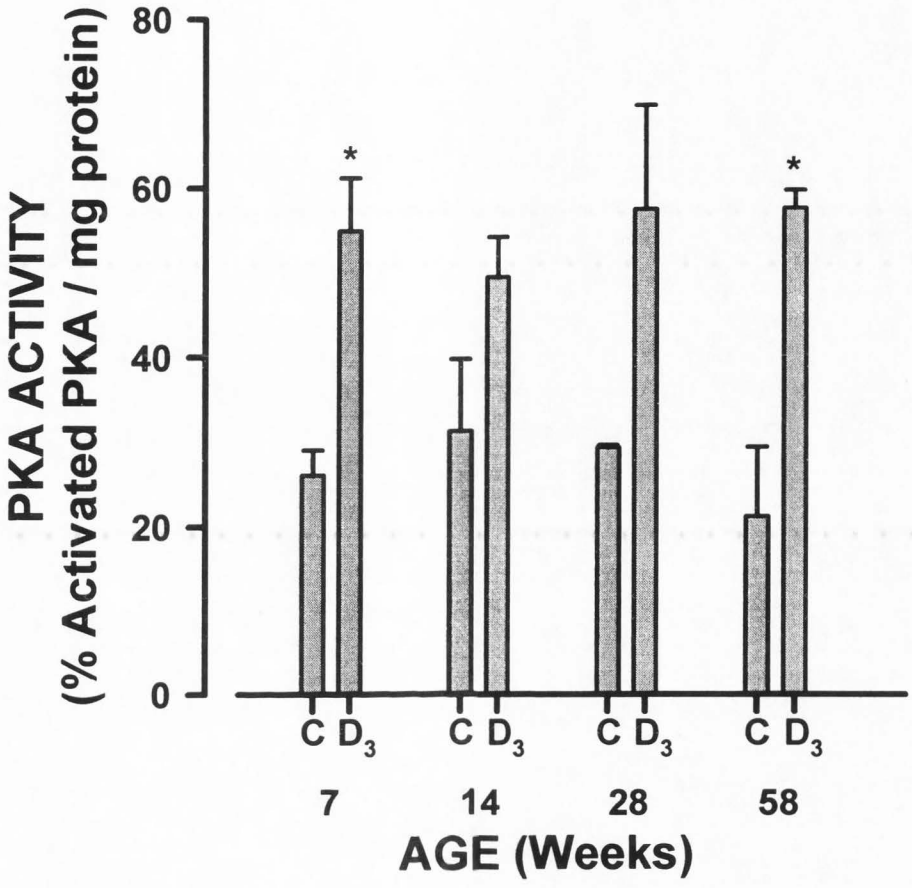

Fig. 2.10. PKA activity in male chicken enterocytes. Intestinal epithelial cells from 7-, 14-, 28-, and 58-week-old male chickens were isolated and treated with either 130 pM $1,25(OH)_2D_3$ (D_3) or ethanol , 0.01 % final concentration (C) for 7 min, the cells harvested by centrifugation, extracted, and the supernatant fractions analyzed for protein kinase A (PKA) activity. Significant differences between D_3 and C within each age group (*) and differences between C for the different age groups (small letters) were obtained by a one-way ANOVA followed by Student's Newman-Keuls post-hoc test ($P<0.05$). Data are presented as mean ± SEM (n = 3-7).

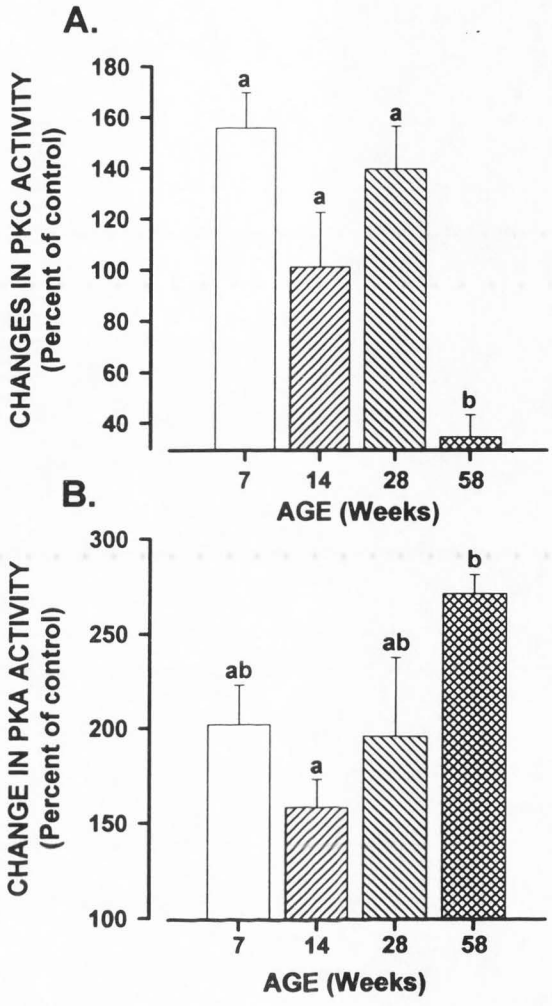

Fig. 2.11. Age related changes in PKC and PKA activities in male chickens. Protein kinase, PKC, (**A**) and protein kinase A, PKA, (**B**) activities in $1,25(OH)_2D_3$-treated cells as percent of vehicle treated cells are presented. Intestinal cells from 7-, 14-, 28-, and 58-week-old male chickens were isolated and treated with either 130 pM $1,25(OH)_2D_3$ or ethanol (0.01 % final concentration). For PKC cells were treated for 5 min and for PKA cells were treated for 7 min. Significant differences between age groups (small letters) were obtained by an one-way ANOVA followed by Student's Newman-Keuls post-hoc test. Data are presented as mean ± SEM and $P<0.05$ for significance. For PKC n = 4-14 and for PKA n = 3-7 independent cell preparations for each age group tested.

intestinal calcium transport shows a decrease with increasing age in male chickens, which is in agreement with earlier results in mammals (9, 10). This is the first demonstration that the membrane-initiated, rapid effects of $1,25(OH)_2D_3$ on calcium transport may be of greater physiological significance in young, growing animals, than in mature animals.

Binding analyses of the hormone to nVDR and 1,25-MARRS bp were performed to investigate which hormone-mediated pathway is affected by increasing age, and may contribute to the reduced hormone responsiveness observed. The results of these studies should be interpreted within the context of serum levels of $1,25(OH)_2D_3$. For a receptor to give maximal respond to hormone stimulation, the receptor needs to have a K_d close to circulating hormone levels. If a receptor has a K_d much greater than physiological hormone levels the receptor will have too low affinity for the hormone to give an optimal response to the stimulus. Accordingly, a receptor having a K_d value much lower than circulating hormone levels will have too high affinity to the hormone to be able to respond. Thus, for a receptor to give an optimal response to hormone activation it needs to have a K_d-value close to circulating hormone levels. The number of unoccupied binding sites (B_{max}), also depends on the circulating hormone levels. As the hormone levels increase B_{max} is expected to decrease, if the total number of receptors is the same. Consequently, if no new receptors are synthesized a decreased hormone level is reflected by increased B_{max}. As reported by Horst and Littledike (33), young chickens (6-12 weeks of age) have higher levels (0.27 nM hormone), than adult animals (>40 weeks of age), where serum levels drop to 0.05 nM steroid.

Curve-fitting analyses of data obtained for $[^3H]1,25(OH)_2D_3$ binding to nVDR,

yielded a hyperbolic function, and Hill analysis of the data indicated an n_{app} not significantly different from 1, indicating an absence of cooperative binding. No changes in K_d or B_{max} were observed with increasing age. A number of studies have shown a decrease in both nVDR protein and mRNA levels in intestine with increasing age (20-23), which have been proposed to be one factor causing the observed age-related decrease in $1,25(OH)_2D_3$ responsiveness. However, our results showed no changes in B_{max}, which is the maximal binding capacity of the receptor, indirectly indicating no changes in number of unoccupied receptors. Results from studies in rats and humans by Wood *et al.* (10) and Kainyamu *et al.* (3), respectively, are in agreement with our present findings. With respect to the affinity of the receptor, it has been shown by others to be unaffected by age (20, 21), which agrees with our results showing no significant changes in K_d with increasing age, remaining at approximately 0.3 nM $1,25(OH)_2D_3$. This lack of change makes it unlikely that the nVDR is responsible for mediating the rapid stimulation of calcium transport that declines with age. Although serum levels of $1,25(OH)_2D_3$ fall to 0.05 nM after 40 weeks (33), it is conceivable that sufficient transcriptional activity by the nVDR continues to maintain components of the calcium transport pathway (see also below). Erben *et al.* (34) have reported studies on mice engineered to contain an nVDR lacking the DNA binding domain, but retaining the ligand binding domain, which is phenotypically equivalent to VDR -/- mice. They argue that such a mutation should not affect the $1,25D_3$-MARRS bp, and that therefore only the nVDR is necessary for nuclear and membrane-initiated effects (34). However, the authors did not consider the earlier observations that the nuclear actions of $1,25(OH)_2D_3$ are required to induce components of the calcium transport pathway (including signal transduction—see below,

microtubules, and transport vesicles [12]), which allow ligand binding to the $1,25D_3$-MARRS bp to produce an effect.

In the current work, [^{3}H]$1,25(OH)_2D_3$ saturation binding analyses of the $1,25D_3$-MARRS bp resulted in data fitting a sigmoid function, while Hill analysis gave n_{app} significantly greater than 1. These results reveal a presence of allosteric interactions and positive, cooperative binding. Positive cooperativity of $1,25(OH)_2D_3$ binding to a membrane receptor has previously been reported in the rat osteosarcoma cell line UMR-106 and in the mouse derived osteoblast-like cells, MC-3T3-E1 by Boyan *et al.* (35) and in carp intestinal cells by Nemere *et al.* (36). The observed positive cooperative binding is not restricted to $1,25(OH)_2D_3$ binding to membrane receptors. The steroids, $24,25(OH)_2D_3$ (37), estradiol (38-40) and progesterone (41) have also been demonstrated to bind to plasma membranes with positive cooperativity. The increase in n_{app} with age indicated an increase in cooperative binding. Our data however do not give any information on the nature of the cooperative receptor-hormone binding; that is, whether it is due to interactions of a number of receptor subunits or due to interaction with some other component.

An age-related increase in K_d was found for $1,25D_3$-MARRS bp, indicating a decreased affinity for $1,25(OH)_2D_3$. This diminished affinity may explain the age-related decrease in intestinal responsiveness to $1,25(OH)_2D_3$, especially when combined with lower serum levels of steroid hormone. The latter observation on serum levels of hormone may, however, be off-set in part by the increased positive cooperativity. Calculation of the number of unoccupied binding sites, B_{max}, revealed a significant increase between 7- and 14-week-old birds while there was no difference between 7- and

28-week-old birds. An unequivocal explanation for the observed results is not available. However, the observed increase in B_{max} at 14 weeks may be related to sexual maturation; alternatively, the B_{max} in 7-week-old birds may be underestimated in the current work. Previously, a B_{max} of 250 fmol/mg protein was reported (18) in young birds. In the binding analyses of 58-week-old birds saturation was not obtained within the range of concentrations tested. SDS-PAGE followed by Western analysis revealed a significant linear decrease in number of $1,25(OH)_2D_3$ receptors with increasing age. Thus, the decrease in the high affinity component, $1,25D_3$-MARRS bp, allows binding to lower affinity, high capacity moieties, to yield the observed binding curve. The decreased, but not absent levels of the $1,25\text{-}D_3$-MARRS bp, are consistent with the diminished, but not abolished, levels of $1,25(OH)_2D_3$-stimulated calcium transport.

Aside from receptor levels and affinity, another important point to consider in the aging process, as it relates to steroid-stimulated calcium transport, is the presence or absence of signal transduction pathways.

PKC, which is a member of a family of phospholipid-dependent serine-threonine kinases, is known to play a key role in regulation of cell growth, differentiation and ion channel modulation (42). In rat duodenum, there is an age-related impairment of $1,25(OH)_2D_3$-enhanced phospholipase C production of the second messengers IP_3 and DAG (25), and an altered hormone regulation of calcium transport through the PKC messenger system (26). The sub-cellular distribution of PKC isoenzymes in rat duodenum is also influenced by age (43). The results obtained in the current study, demonstrated that $1,25(OH)_2D_3$-induced PKC activity decreased with age is in agreement with earlier reports (25, 26), and may explain the observed decline in responsiveness of

intestine to $1,25(OH)_2D_3$ with senescence. Moreover, our work demonstrates an age-related decrease in basal PKC activity. Changes in basal PKC activity with senescence have also been reported in rat duodenum, by Balogh *et al* (26), who contrary to our findings, showed an increased activity. At present, we speculate that the differences are species related.

In addition to PKC, PKA has been proposed to stimulate intestinal calcium transport, perhaps in part by activating voltage-gated calcium channels at the basal lateral membrane. This activation in turn leads to a transient increase in intracellular calcium which may activate exocytosis of calcium-containing vesicles, as well as calcium efflux by the calcium pump and the Na^+/Ca^{2+} exchanger, resulting in a net increase in duodenal calcium transport (15, 27, 44, 45). In the current work, $1,25(OH)_2D_3$ treatment of isolated intestinal epithelial cells resulted in an induction of PKA activity in all age groups tested. The induced PKA activity was significantly higher than controls in 7- and 58-week-old birds. In contrast to Massheimer *et al.* (24), who have reported an age-related decrease in the stimulation of cAMP/protein kinase A-dependent calcium uptake by $1,25(OH)_2D_3$ in rat duodenum, our study showed a lower induction of PKA activity in 14-week-old birds than 28-week-old birds. No differences in $1,25(OH)_2D_3$ induced PKA activities were observed when comparing 7-, 28-, and 58-week-old birds. It has earlier been reported that $1,25(OH)_2D_3$ does not induce PKA activity in chickens raised on vitamin D-deficient diet (16). Furthermore treatment of duodena from vitamin D deficient rats with the cAMP derivative, dbcAMP, results in an increase in calcium transport only in intestine pretreated with vitamin D_3 (46). Thus the conclusion that vitamin D is necessary to stimulate the production of components in the PKA signaling pathway was made (16,

46). The continued presence of $1,25(OH)_2D_3$-enhanced PKA activity in the present work suggests that the nVDR remains able to stimulate production of these components with aging. Unimpaired PKA stimulation by the seco-steroid apparently allows the diminished, but not abolished enhancement of calcium transport in mature animals, whereas the contribution of the PKC pathway in young animals leads to a more robust absorption.

In conclusion, this study demonstrates that there is an age-dependent decrease in intestinal calcium uptake as response to exogenous $1,25(OH)_2D_3$ in duodena of male chickens. This decrease is accompanied by a decreased affinity of $1,25D_3$-MARRS bp for $1,25(OH)_2D_3$, a reduced expression of $1,25D_3$-MARRS bp and a decreased PKC activity. In addition, this study gives further proof for the existence of a membrane associated $1,25(OH)_2D_3$ receptor having different physiochemical properties from the classical nVDR. Finally, a physiological importance of the membrane receptor in senescence related changes in responsiveness to $1,25(OH)_2D_3$ in intestine has been demonstrated.

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CHAPTER III

EFFECT OF GROWTH AND MATURATION ON MEMBRANE-INITIATED ACTIONS OF 1,25-DIHYDROXYVITAMIN D_3. II. CALCIUM TRANSPORT, RECEPTOR KINETICS, AND SIGNAL TRANSDUCTION IN INTESTINE OF FEMALE CHICKENS[3,4]

Abstract

This study compares development and maturation in female chickens with the magnitude of membrane-initiated responses to $1,25(OH)_2D_3$ in duodena from 7-, 14-, 28-, and 58-week-old birds. The magnitude of increased intestinal calcium transport, in response to $1,25(OH)_2D_3$ (130 pM), decreased with maturation. Analyses of saturable binding of $[^3H]1,25(OH)_2D_3$ to the membrane associated rapid response steroid binding protein ($1,25D_3$-MARRS bp) in basal lateral membranes (BLMs), showed cooperative binding, and a significant increase in both B_{max} and K_d with increasing age. Furthermore, expression of $1,25D_3$-MARRS bp increased with age, as judged by Western analyses, supporting results obtained in the binding analyses. Saturation analyses of $[^3H]1,25(OH)_2D_3$ binding to nuclear fraction nVDR, indicated an absence of cooperative binding and indicated no significant changes in dissociation constant, K_d or number of unoccupied receptor binding sites, B_{max} with age. Protein kinase C (PKC) activity, both in controls and in response to $1,25(OH)_2D_3$, decreased with age. Protein kinase A (PKA)

[3]Coauthered by Birgitta Larsson and Ilka Nemere.
[4]Will be submitted for publication in Am J Physiol.

activity in controls remained constant with age, while responsiveness to $1,25(OH)_2D_3$ increased. Thus, the decrease in responsiveness to exogenous $1,25(OH)_2D_3$ in female chicken duodena as a function of age, may be partially due to a decreased affinity of $1,25D_3$-MARRS bp for $1,25(OH)_2D_3$, and a reduced PKC activity, suggesting that the membrane-initiated pathway of $1,25(OH)_2D_3$ action is important in growing animals.

Introduction

It is well established that 1,25-dihydroxyvitamin D_3, ($1,25(OH)_2D_3$) is a major regulator of calcium homeostasis. The steroid hormone exerts its effects on one of its major target organs, the intestine; by two mechanisms of which the most well studied is mediated by a nuclear receptor. In this pathway $1,25(OH)_2D_3$ binds to the nuclear vitamin D receptor (nVDR) and initiates a cascade of events that affect transcription (1, 2). In addition, the steroid hormone $1,25(OH)_2D_3$ initiates a variety of biological responses in intestine and other tissues too rapidly to be explained by genome activation (3, 4). Evidence has been obtained indicating that these processes are associated with the modulation of cell membrane voltage dependent calcium channels (5-7), as well as increases in the intracellular second messengers cyclic adenosine monophosphate (cAMP), and, protein kinase C (PKC), and protein kinase A (PKA) (7-10). In the enterocyte, activation of these membrane-associated signalling systems results in a rapid enhancement of calcium transport (11). It has been proposed that $1,25(OH)_2D_3$ mediates its rapid effects by interacting with a putative protein receptor, now termed the membrane associated rapid response steroid binding protein ($1,25D_3$-MARRS bp) located in the plasmalemma (12), rather than a nuclear receptor. Specific binding of $1,25(OH)_2D_3$ to

basal lateral membranes has been reported in chicks and antisera has been raised to the N-terminus of the protein (Ab 099; 10, 13). Immunofluorescent staining of intestinal tissue sections with Ab 099 revealed the presence of the protein in the basal lateral membrane, but not brush border membrane (10). Presentation of $1,25(OH)_2D_3$ to its putative receptor by vascular perfusion of isolated duodenal loops results in an over all increase in intestinal calcium transport, observed within minutes after $1,25(OH)_2D_3$ administration, whereas introduction of the steroid to the luminal perfusate, and hence the brush border membrane, does not result in stimulation of transport (14).

It has long been known that aging is accompanied by decreased intestinal calcium absorption in humans and other vertebrates (15-20). The capacity of $1,25(OH)_2D_3$ to stimulate calcium absorption also declines with age (16, 17, 21), but the biochemical mechanisms responsible for this decrease in intestinal function are not fully understood. A decrease in expression of nVDR indicating the involvement of the classical pathway has been reported (1, 22-25). However, it has also been shown that enterocytes isolated from aged rats exhibit a marked fall in the stimulation of cAMP/PKA-dependent Ca^{2+} influx by $1,25(OH)_2D_3$ in comparison to cells from young animals (26). In addition, the production in inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) stimulated by the $1,25(OH)_2D_3$ significantly decreases with senescence, providing indirect proof for the involvement of the $1,25D_3$-MARRS bp mediated pathway (27, 28). Although evidence exists for the involvement of both pathways, there has been no direct comparison within the same model system.

Thus, the present study was designed to explore age-related changes in $1,25(OH)_2D_3$-stimulated intestinal calcium transport in female chickens which have high

calcium demand both during growth (bone formation), and during adulthood (egg laying). The magnitude of the $1,25(OH)_2D_3$-mediated physiological response was compared to steroid-mediated changes in the intracellular second messenger systems PKC and PKA, as well as changes in receptor affinity and maximal binding capacity of the classical nuclear receptor, nVDR, and the $1,25D_3$-MARRS bp.

Materials and methods

Materials

Female white Leghorn chicks were from Merrill Poultry, Poul, ID. Vitamin D-supplemented diet (1.0 % calcium, 1.0 % phosphorus) was from Nutrena Feeds (Murray, UT), chloropent from Fort Dodge Laboratories (Fort Dodge, IA), $^{45}CaCl_2$ and $[^3H]1,25(OH)_2D_3$ were from NEN Life Science Products Inc (Boston, MA). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Bradford, MA), kits for PKC and PKA determination were from Life Technologies-GIBCO (Waverly, MA), Bradford dye was from Bio-Rad (Hercules, CA) and $[\gamma\text{-}^{32}P]$ATP was purchased from NEN (Boston, MA). All other chemicals were of highest grade available and obtained from Sigma Chemical Co (St Louis, MO).

Animals

White Leghorn pullets, were obtained on the day of hatch and raised on a vitamin D-supplemented diet prior to experimentation. Animals of ages 7, 14, 28, and 58 weeks were studied. Growth phases were represented by 7- and 14-week-old birds. Since onset of egglaying generally occurs at week 20, mature hens were represented by 28- and 58-

week-old birds. On the day of the experiments, chickens were anesthetized with 0.3 ml chlorohydrate sodium pentabarbital/100g B.W.

Perfusion studies

Perfusion studies were performed as described earlier (29). In brief, luminal perfusion was performed with Gey´s balanced salt solution (GBSS), lacking bicarbonate and containing 1 μCi $^{45}CaCl_2$/ml. Each perfusion experiment was divided into three periods (Fig. 3.1). During the first period (10 min), vascular perfusion with aerated GBSS-control medium (containing 0.005 % ethanol, final concentration) was conducted to allow the system to reach a steady state. During the second period (10 min) samples of venous effluent are collected for assessment of basal ^{45}Ca transport. Throughout the third period the preparation was exposed to either GBSS-control medium (pefusion of 7-, 14-, 28-, and 58-week-old birds n = 3, 4, 6, and 5 respectively) or 130 pM $1,25(OH)_2D_3$ in GBSS (pefusion of 7-, 14-, 28-, and 58-week-old birds n = 5, 6, 7, and 4, respectively) for 40 min and radioactivity assessed in the collected samples. The transport during the third period was normalized to the corresponding average basal transport rate according to following formula:

$$\frac{cpm_t = T_{(0-40)}}{\sum_{t=-10}^{T=0} cpm/5}$$

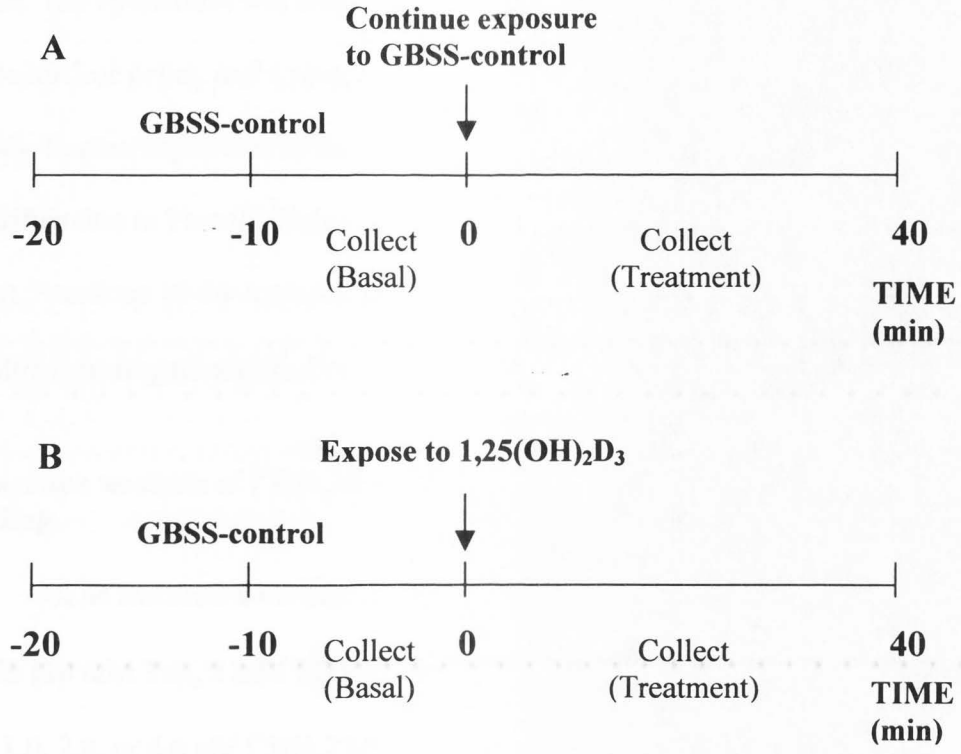

Fig. 3.1. Experimental design of perfusion studies on duodena of female chickens. Duodenal loops from female chickens of ages 7, 14, 28, or 58 weeks were vascularly perfused with either GBSS-control media or 130 pM $1,25(OH)_2D_3$ in GBSS. (**A**) shows the experimental design for perfusion with GBSS-control medium and (**B**) represents perfusion with 130 pM $1,25(OH)_2D_3$. In both control and $1,25(OH)_2D_3$ treatment the duodena were vascularly perfused with GBSS-control medium between –20 to 0 min. Venous effluent was collected at time –10 to 0 min for assessment of basal ^{45}Ca transport. At time 0 min the vascular perfusion with GBSS-control medium was either continued for 40 min (**A**) or the treatment with GBSS-control medium was stopped and the animals were instead vascularly perfused with $1,25(OH)_2D_3$ in GBSS (**B**). Aliquots of venous effluent were collected between time 0 to 40 min (treatment period) and ^{45}Ca assessed.

Preparation of crude nuclei and basal lateral membranes

Subcellular fractions were prepared as reported earlier (13, 30, 31). Intestinal epithelium was disrupted in 40 ml homogenization medium (250 mM sucrose, 5 mM histidine-imidazole, 2 mM EGTA, pH 7.0) with a Dounce homogenizer and a teflon

pestle. The epithelium was fractionated by differential centrifugation, resulting in a crude nuclear pellet, and a postnuclear pellet containing intracellular organelles plus BLMs. Further separation of the organelles and membranes was achieved by centrifugation in Percoll. Eighteen fractions were collected from the gradient (52 drops each). Fractions 16-18 containing BLM (13, 30, 31) were pooled and the Percoll removed by ultra centrifugation (13). Crude nuclei and BLM were stored at –20 °C until analysis.

Saturation analysis of [^{3}H]1,25(OH)$_2$D$_3$ binding

BLM fractions were adjusted to 50 μg of protein per tube and incubated in TED buffer (10 mM Tris, 2 mM EDTA, 1 mM dithiothreitol, pH 7.4) over night (0 °C) with 0.5, 1.0, 2.0, or 4.0 nM [^{3}H]1,25(OH)$_2$D$_3$ in the absence (total binding) or in the presence of a 200 fold molar excess of unlabeled 1,25(OH)$_2$D$_3$ (nonspecific binding). Each sample was assayed in triplicate for total and nonspecific binding. Nuclear fractions from each group were incubated in the same way as BLMs, but with [^{3}H]1,25(OH)$_2$D$_3$ concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, or 2.0 nM.

The following morning, bound and free ligand were separated by either perchloric acid precipitation for binding to BLM (13) or by a hydroxyapatite (HAP) assay for binding to nuclear fractions (32).

For BLM fractions, perchloric acid and carrier protein (γ-globulin) were added to each tube, the mixture was incubated on ice for 20 min, and the precipitated protein pelleted by centrifugation. The pellets containing the seco-steroid binding moiety were solubilized in guanidinium/HCl, decanted into vials, liquid scintillation cocktail added, and radioactivity determined.

While the perchloric acid method is suitable for hydrophobic membrane proteins (1,25D_3-MARRS bp), it fails to detect the soluble classical vitamin D receptor, nVDR (13). Binding of [^{3}H]1,25$(OH)_2D_3$ was therefore assessed in crude preparations of nuclei by the use of a HAP assay as reported elsewhere (32). After incubation with different concentrations of [^{3}H]1,25$(OH)_2D_3$ over night (see above), 200 µl HAP and 800 µl TED containing 0.5 % Triton X-100 were added to each tube, and mixed. The HAP-bound receptor was pelleted by centrifugation, washed twice with 800 µl TED containing 1.5 % Triton X-100, treated with 1 ml ethanol to extract ligand, and centrifuged. The resulting supernatant fraction was transferred to glass scintillation vials, the ethanol evaporated and the amount [^{3}H]1,25$(OH)_2D_3$ measured after addition of fluor.

SDS-PAGE and Western analysis

SDS-PAGE and Western blot analysis were used to determine immunoreactive levels of 1,25D_3-MARRS bp. Proteins (15 µg/ well) were separated on an 8 % sodium dodecylsulfate (SDS) polyacrylamide gel with a 5 % stacking gel. After separation on SDS-PAGE, proteins were transferred to a PVDF membrane (Immobilon P, Millipore) by the use of a Trans-Blot SD Semi-dry transfer cell (Bio Rad) and Western analyses were performed according to the Millipore protocol as follows. To avoid non-specific binding, the membrane was soaked for 1 h at 37 °C in blocking solution, 0.5 % non-fat dry milk in phosphate buffered saline, PBS (PBS; 0.9 % NaCl, 10 mM Na_2HPO_4, pH 7.4), followed by washing 3 × 5 min with washing solution, 0.1 % (w/v) BSA in Tris buffered saline (TBS;0.9 % NaCl in 20 mM Tris-HCl pH 7.4), and incubation with primary antibody Ab099 (rabbit anti-1,25D_3-MARRS bp N-terminal peptide; dilution 1/5000 in TBS

containing 1 % BSA, 0.05 % Tween-20) (10) over night at 4 °C. After three additional 3 × 5 min washes, the membrane was incubated with secondary antibody (alkaline phosphatase conjugated goat anti rabbit IgG), in TBS with 1 % BSA, 0.05 % Tween-20 for 2 h at room temperature and, then washed as before. Immunoreactive bands were visualized with the chromogens, BCIP/NBT and relative amounts of $1,25D_3$-MARRS bp were quantitated by using densitometry and computer software.

PKC and PKA activity and protein determination

Intestinal epithelial cells from two duodena per experiment were isolated by citrate chelation as reported elsewhere (33). After removal of the duodenal loop and chilling for 15 min in saline, the pancreas was excised, the duodenum was everted, rinsed in saline, and the intestinal epithelial cells isolated by stirring the segments in chelation media for three, 15-min periods. The isolated cells were combined, centrifuged at 500 × g, 4 °C for 5 min and the resulting pellet resuspended in 20 ml GBSS-BSA (0.125% BSA, w/v). Cells were treated at room temperature with either ethanol (0.005 %, final concentration) or 130 pM $1,25(OH)_2D_3$ at time zero. For PKC, cells were incubated for 5 min, and for PKA the incubation lasted for 7 min (34). After incubation the cells were centrifuged at 1000 × g, 4 °C for 10 min, the supernatant decanted. The inside of the tube swabbed with Kinwipe and the pellet stored at –20 °C until use.

PKC and PKA activities were analyzed by the use of commercially available assay systems (Appendix B). The analyses and activity calculations were performed according to instructions packaged with the kits. Briefly, enzyme activity was extracted by homogenizing treated cells in the appropriate extraction buffer, followed by

incubation on ice for 30 min, and centrifugation. Supernatants were analyzed for PKA or PKC activity. For PKC activity 10 μgs of extracted protein were incubated at room temperature for 20 min in presence of PKC activator or inhibitor. [γ-^{32}P]ATP (20-25 μCi/ml) was added to each tube, incubated for 5 min at 30 °C, and 25 μl of the mixture spotted onto a phosphocellulose disc. The discs were washed, transferred to scintillation vials, incorporated ^{32}P assessed, and specific PKC activity calculated. For PKA activity, extracted proteins (5 μg) were incubated at room temperature for 20 min in presence of activator or inhibitor only, in presence of both activator and inhibitor and in absence of both. [γ-^{32}P]ATP (20-25 μCi/ml) was added to each tube, the tubes incubated for 5 min at 30 °C, and 20 μl of the mixture spotted onto phosphocellulose disc. The discs were washed, transferred into scintillation vials, ^{32}P assessed, and PKA activity calculated.

Proteins were determined using the Bradford dye (BioRad) according to manufacturers instructions (Appendix B).

Statistics and data analysis

Data available from perfusion studies (I. Nemere, unpublished observations) were analyzed by Student's t-test for unpaired observations within each age group, between treated and controls. For trend analysis between age groups, linear regression was performed with values of T/Av basal at 40 min (see below).

The specific binding of [^{3}H]1,25$(OH)_2D_3$ to receptor was calculated and plotted against the corresponding concentration of [^{3}H]1,25$(OH)_2D_3$ The data were analyzed by nonlinear regression analysis by fitting either to a three parameter sigmoid equation or a hyperbolic function.

The sigmoid equation was as follows:

$$Y = \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

where Y is specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (fmol/mg protein), x is the concentration of 1,25(OH)$_2$D$_3$ (nM) in the incubation mixture, a is the maximum specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (B_{max}), b is the minimum specifically bound [^{3}H]1,25(OH)$_2$D$_3$ and x_0 is the concentration of 1,25(OH)$_2$D$_3$ in the incubation mixture at half maximum bound [^{3}H]1,25(OH)$_2$D$_3$ (K_d).

For the hyperbolic function the equation was as follows:

$$Y = \frac{a * x}{b + x}$$

where Y is specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (fmol/mg protein), x is the incubation concentration of 1,25(OH)$_2$D$_3$ (nM) in the incubation mixture, a is the maximum specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (B_{max}) and b is the concentration of 1,25(OH)$_2$D$_3$ in the incubation mixture at half maximal bound [^{3}H]1,25(OH)$_2$D$_3$ (K_d).

Data from Hill analysis, perfusion studies and PKA and PKC activities were analyzed by linear regression. The coefficient of determination (R^2) and the adjusted coefficient of determination ($_{adj}R^2$) were used as a measure of how well the regression model describes the data. A one-way analysis of variance (ANOVA) with F-statistics was used to gauge the contribution of the independent variable to predict the dependent variable. The significance level was set at $P<0.05$, and data are presented as mean ± SEM.

When comparing B_{max}, K_d, and Hill coefficients, between different age groups an

unpaired Student's t-test was used. When variables were used in more than one comparison, a sequentially rejective Bonferroni test (35) was used. In the PKC, PKA, and Western blot analysis statistical comparisons were performed using one-way ANOVA followed by Student's Newman-Keuls posthoc test when comparing a factor with more than two levels. The tests used were two-tailed, and the significance level was set at $P<0.05$. Data are presented as mean ± SEM.

Results

Effects of age on intestinal calcium transport

As a test of physiological responsiveness to hormone, calcium transport was studied in isolated, perfused duodena. Female chickens of ages 7, 14, 28, and 58 weeks having average weights (mean ± SEM) of 0.33 ± 0.06, 0.91 ± 0.02, 1.47 ± 0.06, and 1.71 ± 0.04 kg, respectively, were used. Basal calcium transport was determined to be 1.6 ± 0.5, 1.5 ± 0.2, 1.7 ± 0.3, and 0.7 ± 0.2 μg ^{45}Ca in venous effluent/min for 7-, 14-, 28-, and 58-week-old birds, respectively. No significant changes in basal calcium transport with age were observed.

For stimulation of calcium transport, 130 pM $1,25(OH)_2D_3$ was used in the vascular perfusate, and compared to vehicle controls. As shown in Fig. 3.2A-D, treatment with 130 pM $1,25(OH)_2D_3$ resulted in a linear increase in intestinal calcium transport. Hormone induced calcium transport became significantly higher than controls at T = 8, T= 6, T = 16, and T = 34 min for 7-, 14-, 28-, and 58-week-old birds, respectively. At 40 min, 7-, 14-, 28-, and 58-week-old birds attained treatment/basal ^{45}Ca values in venous

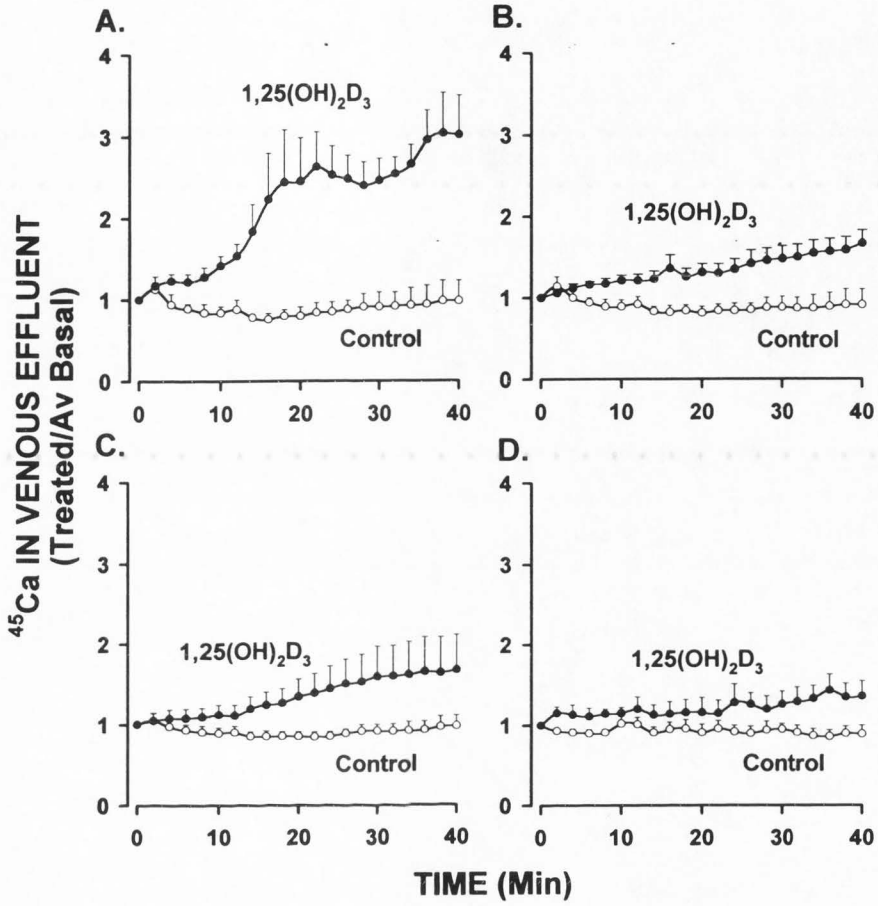

Fig. 3.2. Effects of age on the rapid stimulation of intestinal calcium transport by $1,25(OH)_2D_3$ in female duodena. Isolated duodena of female chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were vascularly perfused with vehicle (0.005 % ethanol, final concentration) during the basal phase, and again in the treated phase for controls (open symbols), or with 130 pM $1,25(OH)_2D_3$ during the treated phase (closed symbols). Values represent ^{45}Ca in the venous effluent at T min divided by average basal cpm. Data are expressed as mean ± SEM (n = 3-7 for each treatment within each age group).

effluent of 3.0 ± 0.5, 1.7 ± 0.2, 1.7 ± 0.4, and 1.4 ± 0.2 respectively, while the controls were 1.0 ± 0.3, 0.9 ± 0.2, 1.0 ± 0.1, and 0.9 ± 0.1, respectively (Fig. 3.2). Accordingly, stimulated calcium transport in duodena from 7-, 14-, 28-, and 58-week-old birds went from 308 % of controls to 184 %, 170 %, and 153 % of controls, respectively, at T = 40 min.

Linear regression analysis showed an age-dependent decrease in stimulated calcium transport when comparing ^{45}Ca in the venous effluent (cpm at T = 40 min) / (Average basal cpm) for the different ages ($P<0.05$, F = 7.2 DF = 21, $R^2 = 0.27$, and $_{adj}R^2 = 0.23$) (Fig. 3.3).

Saturation analysis of $1,25(OH)_2D_3$ binding to nVDR

Figures 3.4A-D illustrate the results of experiments in which specific binding activity was determined as a function of increasing concentrations of ligand in nuclear fractions prepared from 7-, 14-, 28-, and 58-week-old female birds. Data obtained in the analyses were fitted to a hyperbolic function ($P<0.05$ for all ages, 7 weeks; F = 150, DF = 12, $R^2 = 0.93$, and $_{adj}R^2 = 0.92$, 14 weeks; F = 115, DF = 13, $R^2 = 0.91$, and $_{adj}R^2 = 0.90$, 28 weeks; F = 31, DF = 13, $R^2 = 0.72$, and $_{adj}R^2 = 0.70$, and 58 weeks; F = 77, DF =13, $R^2 = 0.87$, and $_{adj}R^2 = 0.85$). Maximal binding capacities, (B_{max}) for 7, 14, 28, and 58 weeks old birds were calculated to be 19 ± 1.0, 30 ± 2.6, 26 ± 3.0, and 29 ± 2.5 fmol/mg protein, respectively. In the same age groups, specific binding became half-saturable at a K_d of 0.1 ± 0.02, 0.2 ± 0.06, 0.1 ± 0.04, and 0.1 ± 0.04 nM, respectively. No significant differences in K_d with increasing age were observed. The B_{max} for 7-week birds was significantly lower than the B_{max} for 14- and 58-week-old birds. No significant

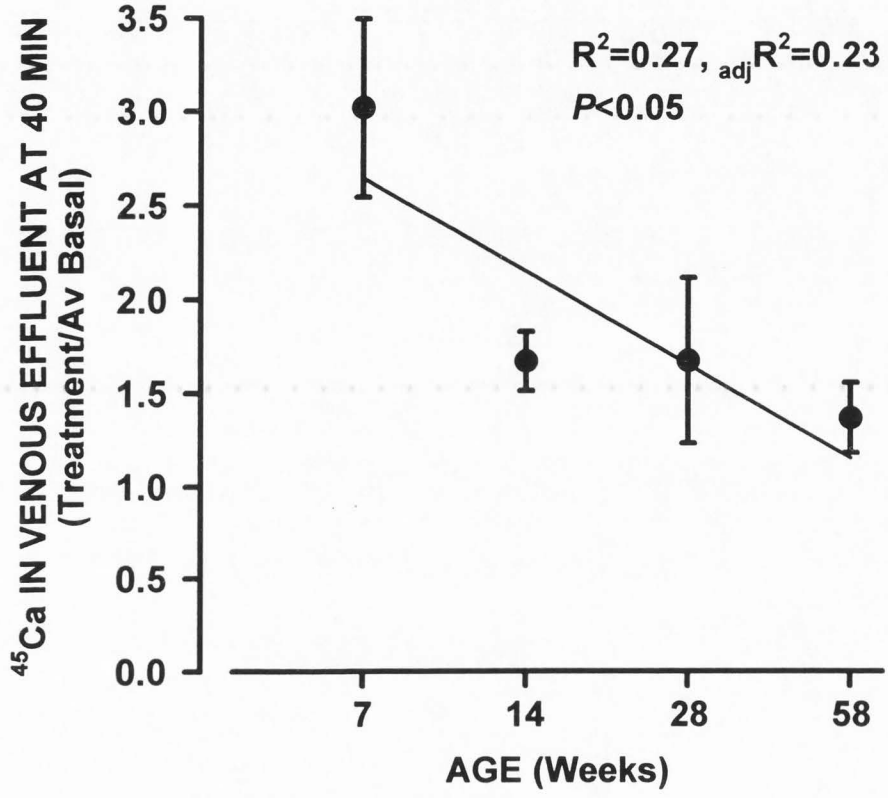

Fig. 3.3. Changes in ^{45}Ca in venous effluent with age in perfused duodena of female chickens. Isolated duodena of female chickens of ages 7, 14, 28, and 58 weeks were vascularly perfused with the vehicle ethanol (0.005%, final concentration) during the basal phase, and in the treated phase with 130 pM $1,25(OH)_2D_3$. Values represent ^{45}Ca in the venous effluent at T = 40 min normalized to the corresponding average basal transport determined at T= -10 to 0 min. A linear regression analysis of the data revealed a significant ($P<0.05$) decrease in $1,25(OH)_2D_3$ induced intestinal calcium uptake with increasing age. R^2 is the coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. Data are presented as mean ± SEM (n = 4-7).

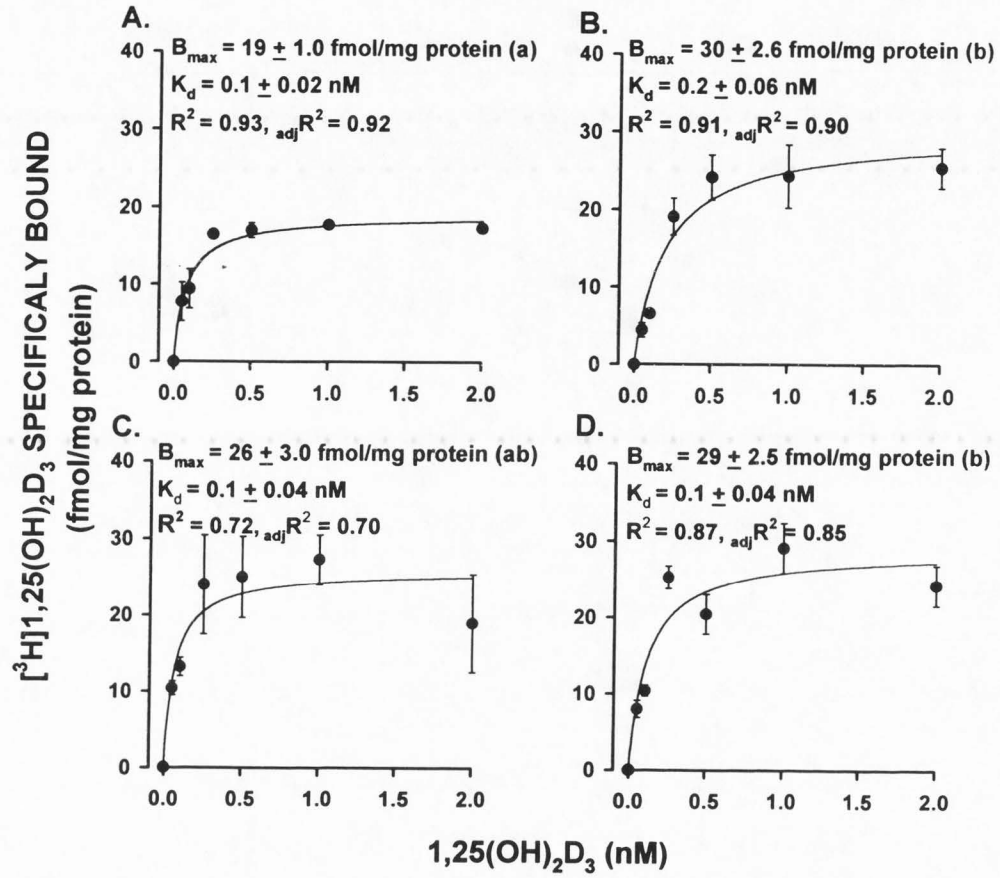

Fig. 3.4. Saturation analysis of $1,25(OH)_2D_3$ binding to nVDR from crude nuclear fractions prepared from mucosa of female chickens. Crude nuclear fractions enriched in nVDR isolated from female chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were incubated with 0.05, 0.1, 0.25, 0.5, 1.0 or 2.0 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or in the presence of a 200-fold molar excess of unlabeled steroid. Bound radioactivity was assessed with the HAP assay. Data were fitted to a three parameter hyperbolic function and tested by nonlinear regression. R^2, coefficient of determination and, $_{adj}R^2$, the adjusted coefficient of determination. Small letters indicate significant differences determined by an unpaired Student's t-test. $P<0.05$ for significance and data are presented as mean ± SEM. Saturation analysis was performed on three independent preparations within each age group.

differences in B_{max} were obtained when comparing 14-, 28-, and 58-week-old birds.

Data presented in Figs. 3.4A-D were recalculated for Hill analyses which yielded curves that could be described by a linear regression ($P<0.05$ for all ages, 7 weeks; F = 33, DF = 10, $R^2 = 0.79$ and $_{adj}R^2 = 0.76$, 14 weeks; F = 42, DF = 11, $R^2 = 0.81$, and $_{adj}R^2 = 0.79$, 28 weeks; F = 6.4 DF = 8, $R^2 = 0.88$ and $_{adj}R^2 = 0.86$, and 58 weeks; F = 60, DF =7, $R^2 = 0.91$, and $_{adj}R^2 = 0.89$). The resultant apparent Hill coefficients (n_{app}) were 0.9 ± 0.2, 1.1 ± 0.2, 0.7 ± 0.1, and 1.0 ± 0.1 for 7-, 14-, 28-, and 58-week-old birds, respectively (Fig. 3.5A-D). No significant difference in n_{app} was observed for the different ages. Taken together, both the hyperbolic distribution and the slope in the Hill analysis indicate absence of cooperative binding.

Saturation analysis of $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS bp and Western analysis

Figures 3.6A-D illustrate the specific binding activity as a function of increasing concentrations of ligand in BLM fractions prepared from intestinal mucosa of 7-, 14-, 28-, and 58-week-old female birds. Data obtained in these analyses were fitted to a sigmoid function ($P<0.05$ and DF = 14 for all ages, 7 weeks; F = 22, $R^2 = 0.79$ and $_{adj}R^2 = 0.75$, 14 weeks; F = 42, $R^2 = 0.87$, and $_{adj}R^2 = 0.85$, 28 weeks; F = 65, $R^2 = 0.92$ and $_{adj}R^2 = 0.90$, and 58 weeks; F = 43, $R^2 = 0.88$ and $_{adj}R^2 = 0.85$). B_{max} values were calculated to be 210 ± 21, 330 ± 30, 290 ± 24, and 460 ± 44 fmol/mg protein, and values of K_d were calculated to be 0.8 ± 0.2, 1.3 ± 0.2, 1.6 ± 0.2, and 1.5 ± 0.2 nM for 7-, 14-, 28-, and 58-week-old birds, respectively. A significant age-related increase in both K_d and B_{max} was observed, indicating a reduced affinity and increased number of binding sites with age.

A recalculation of the data in Figs. 3.6A-D for Hill analysis resulted in curves

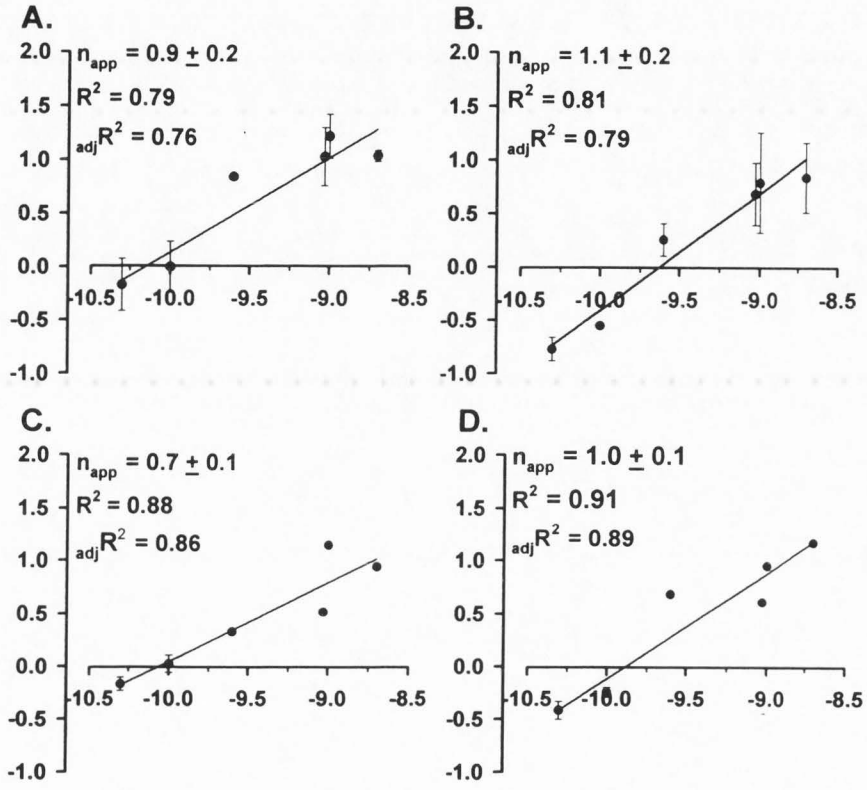

Fig. 3.5. Hill analysis of $1,25(OH)_2D_3$ binding to nVDR in crude nuclear fractions from female chickens. Data presented in Fig. 3.4A-D were recalculated for Hill analysis. (**A**) 7-, (**B**) 14-, (**C**) 28-, and (**D**) 58-week-old birds. [S_b], specifically bound fraction of the administered total free ([S_f]) [^{3}H]$1,25(OH)_2D_3$, R^2, coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. No significant differences in apparent Hill coefficient (n_{app}) were observed with increasing age when compared by an unpaired Student's t-test. An n_{app} close to 1 indicates absence of cooperative binding for $1,25(OH)_2D_3$ to nVDR. Data are presented as mean ± SEM and $P<0.05$ for significance.

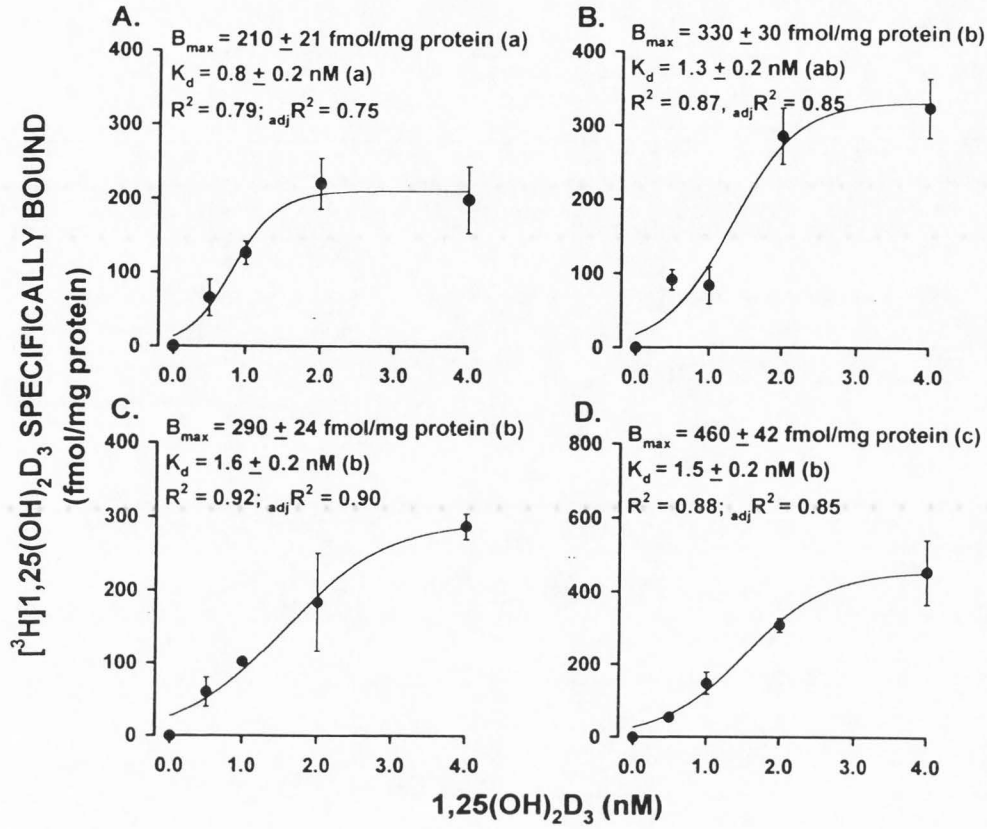

Fig. 3.6. Saturation analysis of $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS bp in three basal lateral membrane (BLM) preparatinos from female chickens. BLM from ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were incubated with 0.5, 1.0, 2.0 or 4.0 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or in the presence of a 200-fold molar excess of unlabeled steroid. Bound radioactivity was assessed by precipitation with perchloric acid in the presence of carrier bovine γ-globulin. Data were fitted to a three parameter sigmoid function and tested by nonlinear regression. R^2, coefficient of determination and $_{adj}R^2$, the adjusted coefficient of determination. Significant differences in maximum binding capacity, B_{max}, and dissociation constant, K_d, with increasing age were determined by Student's t-test. Small letters indicate significant differences ($P<0.05$) between ages. Data are presented as mean ± SEM.

that were described by linear regression ($P<0.05$ for all ages, 7 weeks; F = 33, DF = 7, R^2= 0.70, and $_{adj}R^2$ = 0.65, 14 weeks; F = 12, DF = 8, R^2 = 0.62, and $_{adj}R^2$ = 0.57, 28 weeks; F = 36 DF = 9, R^2 = 0.82, and $_{adj}R^2$ = 0.79, and 58 weeks; F = 38, DF =9, R^2 = 0.83, and $_{adj}R^2$ = 0.81). The Hill analysis yielded Hill coefficients greater than 1 for all ages indicating a presence of positive cooperativity in the binding of $1,25(OH)_2D_3$ to $1,25D_3$-MARRS bp. The n_{app} were calculated to be 2.6 ± 0.5, 1.2 ± 0.4, 1.7 ± 0.4, and 1.6 ± 0.3 for 7-, 14-, 28-, and 58-week-old birds, respectively (Fig. 3.7A-D). Statistical comparison of n_{app} between the ages showed no significant difference.

For each age group, three independent BLM preparations were subjected to SDS-PAGE followed by Western blot analyses of $1,25D_3$-MARRS bp expression. Multiple blots were used for quantitation by densitometric analysis. The resulting data revealed a small, but significant ($P<0.05$) increase in expression of $1,25D_3$-MARRS bp between 28- and 58-week-old birds (Fig. 3.8)

Thus, the results show an increase in both B_{max} and expression of $1,25D_3$-MARRS bp with age, while the affinity of the receptor decreased with maturation. Furthermore, a presence of positive cooperativity in the binding of $1,25D_3$-MARRS bp to $1,25(OH)_2D_3$ has been shown.

Stimulation of PKC and PKA activities

Since differences in intestinal responsiveness to steroid hormones may be due to pleiotropic changes at the cellular level between growing and mature animals, the effects of 130 pM $1,25(OH)_2D_3$ on PKC and PKA activity in isolated intestinal cells from female chickens of different age were tested.

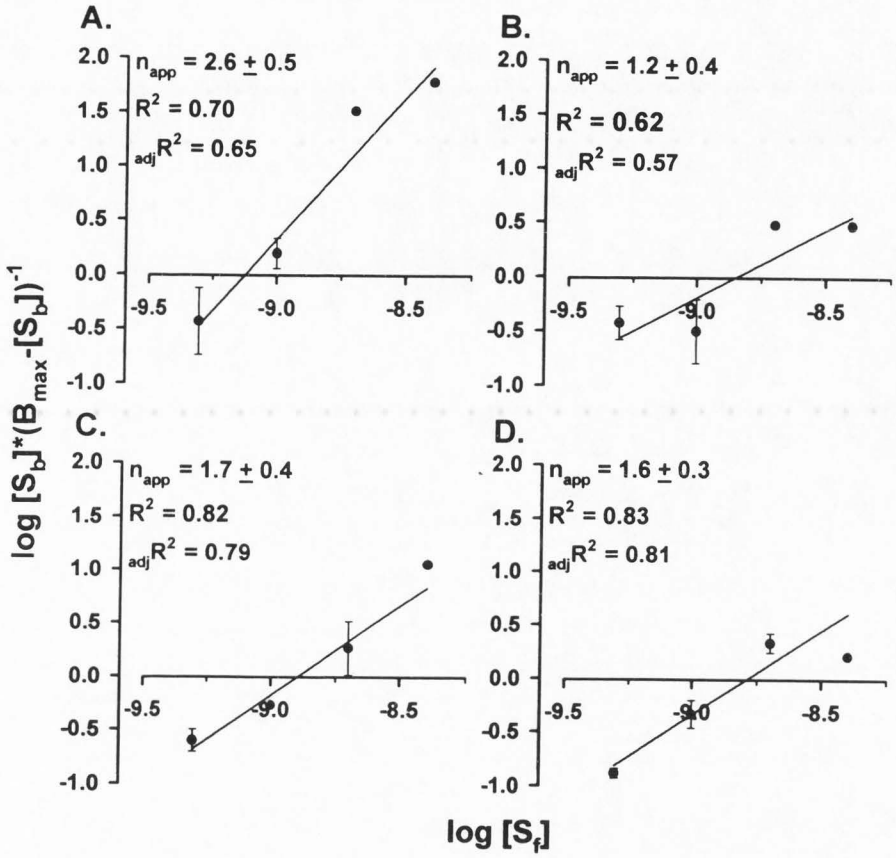

Fig. 3.7. Hill analysis of $1,25(OH)_2D_3$ binding to $1,25D_3$-MARRS bp in BLM from female chickens. Data presented in Fig. 3.6A-D were recalculated for Hill analysis. (**A**) 7-, (**B**) 14-, (**C**) 28-, and (**D**) 58-week-old female birds. $[S_b]$, specifically bound fraction of the administered total free ($[S_f]$) $[^3H]1,25(OH)_2D_3$, R^2, coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. The apparent Hill coefficients (n_{app}) were larger than 1 in all age groups, indicating positively cooperative binding for $1,25(OH)_2D_3$ to $1,25D_3$-MARRS bp. No significant differences in apparent Hill coefficient (n_{app}) were observed with increasing age when compared with a Student's t-test. Data are presented as mean ± SEM.

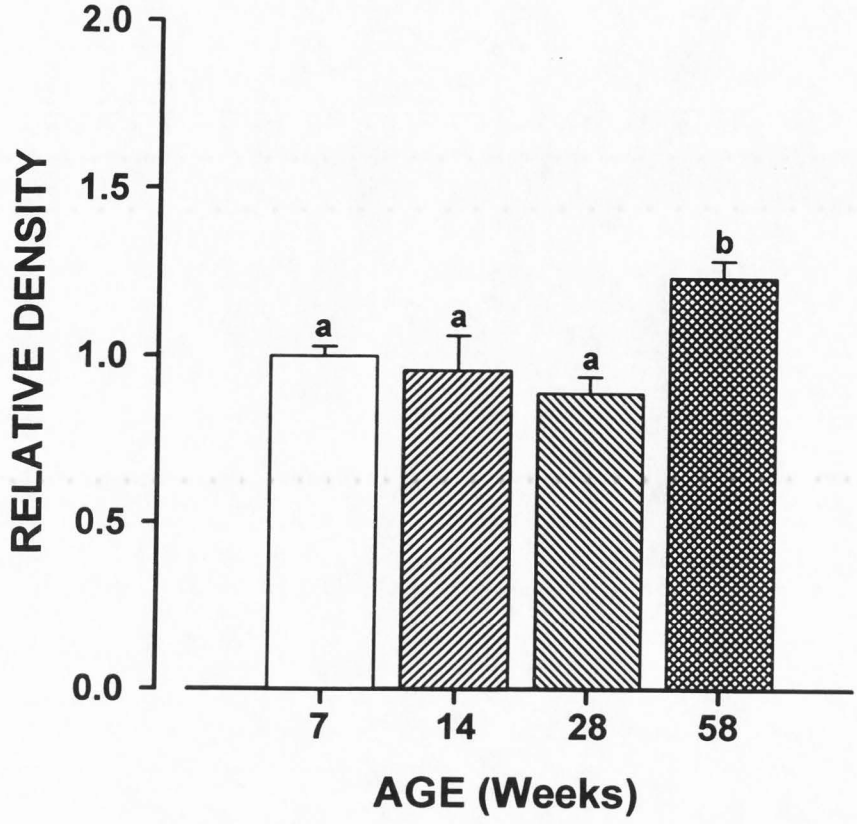

Fig. 3.8. Expression of 1,25D_3-MARRS bp in BLM from female chickens. The expression of 1,25D_3-MARRS bp in basal lateral membranes (BLM) isolated from female chickens of ages 7, 14, 28, and 58 weeks were studied with SDS-PAGE followed by Western blot analyses. BLM were isolated from female chicken duodena and run on 8 % SDS-PAGE. For Western blot analysis Ab099 (rabbit anti-1,25D_3-MARRS bp N-terminal peptide) was used as primary antibody and alkaline phosphatase conjugated goat anti rabbit IgG as secondary antibody. Immunoreactive bands were visualized with the chromogens, BCIP/NBT, and relative amounts of 1,25D_3-MARRS bp were quantitated by using densitometry and computer software. Small letters indicate significant ($P<0.05$) differences between ages obtained by a one-way ANOVA followed by Student's Newman-Keuls posthoc test. Data are presented as mean ± SEM (n = 11) from 3 separate runs.

Figure 3.9 illustrates the results of PKC activity determinations in parallel incubations of control and hormone treated cells from 7-, 14-, 28-, and 58-week-old birds. Extracts from all age groups were assayed concomitantly in order to validate comparisons of basal (control) levels. As shown in Fig. 3.9, PKC activity was affected by age in both vehicle (control) and $1,25(OH)_2D_3$ treated cells. PKC activity in vehicle treated enterocytes yielded values of 57 ± 5.7, 84 ± 14, 130 ± 11, and 70 ± 11 pmol/min/mg protein (n = 3-7) for 7-, 14-, 28-, and 58-week-old birds, respectively, while $1,25(OH)_2D_3$ treatment in the same age groups yielded values of 84 ± 7.7, 94 ± 9.1, 140 ± 12, and 59 ± 2.9 pmol/min/mg protein (n = 3-7), respectively (Fig. 3.9). An apparent increase in basal PKC activity at 14 weeks, which became significant ($P<0.05$) for 28-week-old birds was observed. Basal activity declined again in 58-week-old birds. Upon comparison of $1,25(OH)_2D_3$ treated cells with corresponding controls within each age group a significant ($P<0.05$) increase in PKC activity was observed after treatment with hormone in only 7-week-old animals. The results of analysis of PKA activity in vehicle- and $1,25(OH)_2D_3$-treated cells from 7-, 14-, 28-, and 58-week-old female birds is shown in Fig. 3.10. The activities in vehicle treated cells from 7-, 14-, 28-, and 58-week-old birds were 29 ± 1.5, 29 ± 1.9, 21 ± 1.7, and 32 ± 12 % activated PKA/mg protein (n = 3), respectively, and for $1,25(OH)_2D_3$-treated cells in the same age groups, 47 ± 8.8, 52 ± 12, 54 ± 8.1, and 100 ± 15 % activated PKA/mg protein (n = 3), respectively (Fig. 3.10). Steroid hormone stimulation of PKA activity was suggested in all age groups, though only significant in 28- and 58-week-old birds (Fig. 3.10). PKA activity was unaffected by age in vehicle treated (control) cells.

Figures 3.11A and B show PKC and PKA activities, in $1,25(OH)_2D_3$-treated cells

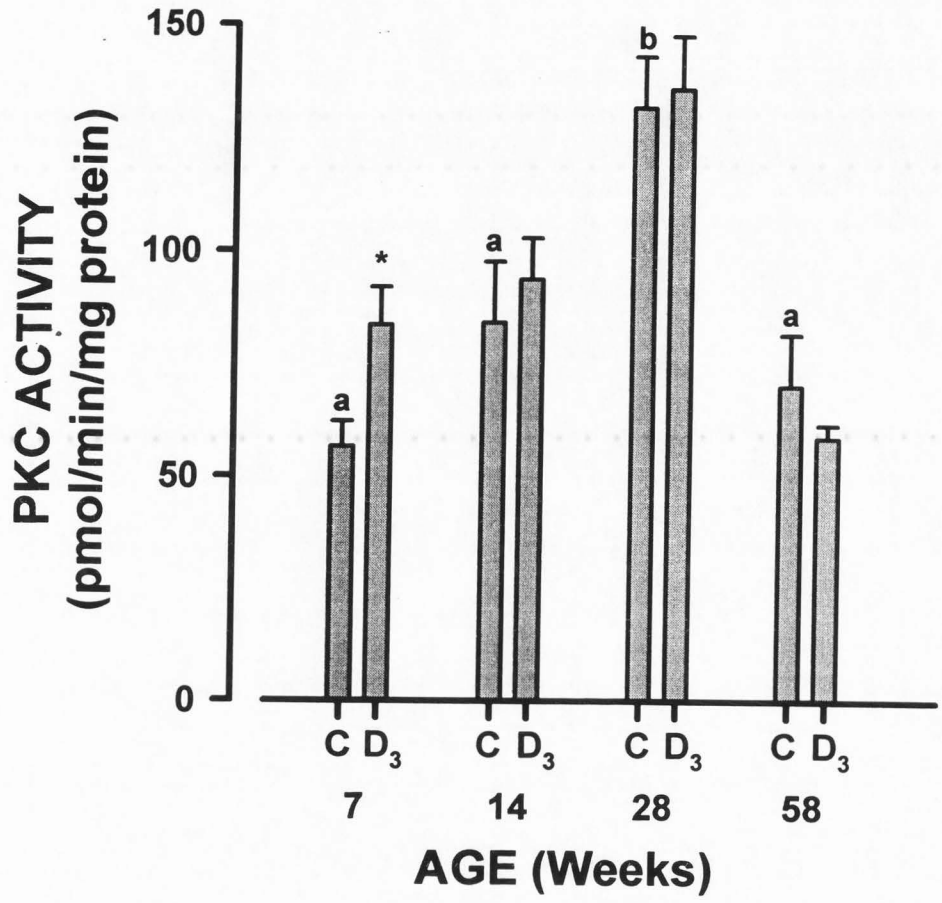

Fig. 3.9. PKC activity in female chicken enterocytes. Intestinal epithelial cells from 7-, 14-, 28-, and 58-week-old female chickens were isolated and treated with either 130 pM $1,25(OH)_2D_3$ (D_3) or ethanol, 0.01 % final concentration (C) for 5 min. The cells were collected by centrifugation, extracted, and the supernatant fractions analyzed for protein kinase C (PKC) activity. Significant differences between D_3 and C within each age group (*) and differences between C for the different age groups (small letters) were obtained by a one-way ANOVA followed by Student's Newman-Keuls post-hoc test ($P<0.05$). Data are presented as mean ± SEM (n = 3-7).

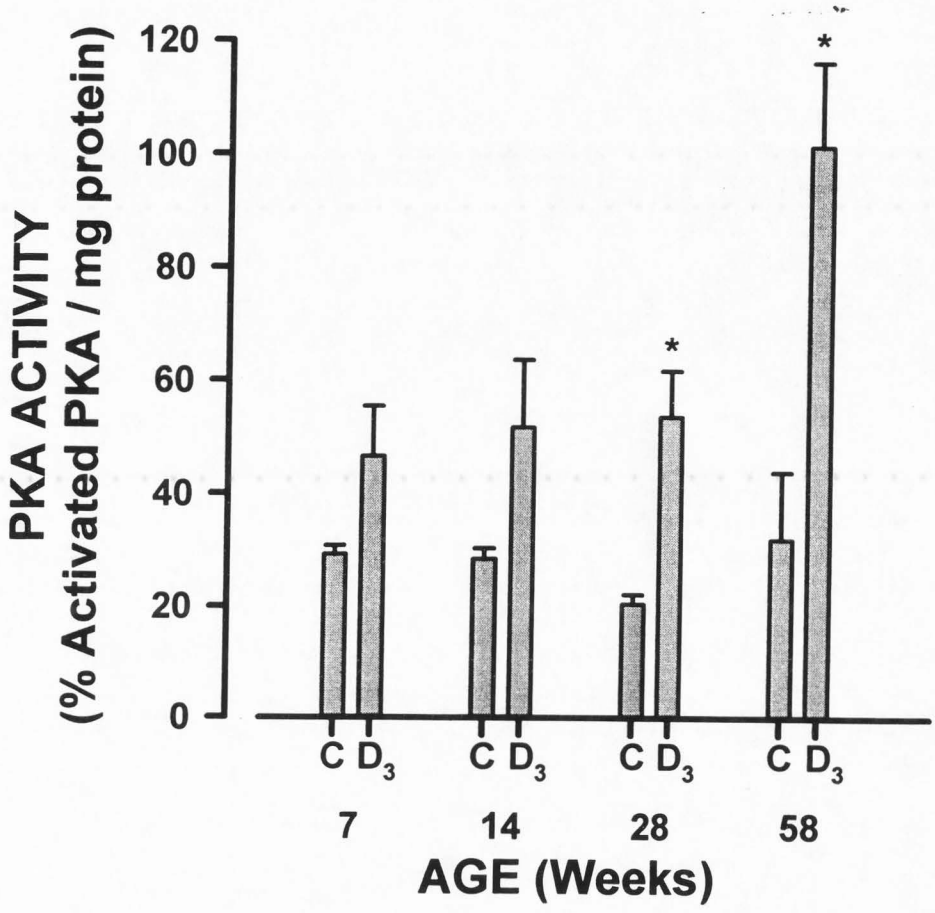

Fig. 3.10. PKA activity in female chicken enterocytes. Intestinal epithelial cells from 7-, 14-, 28-, and 58-week-old female chickens were isolated and treated with either 130 pM $1,25(OH)_2D_3$ (D_3) or ethanol, 0.01 % final concentration (C) for 7 min, the cells harvested by centrifugation, extracted, and the supernatant fractions analyzed for protein kinase A (PKA) activity. Significant differences between D_3 and C within each age group (*) and differences between C for the different age groups (small letters) were obtained by a one-way ANOVA followed by Student's Newman-Keuls post-hoc test ($P<0.05$). Data are presented as mean ± SEM (n = 3).

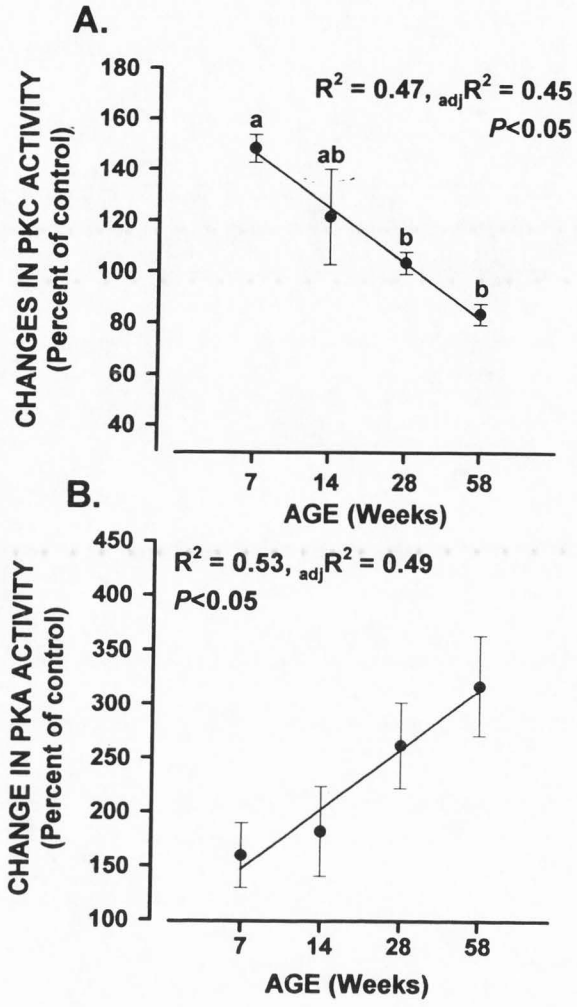

Fig. 3.11. Age related changes in PKC and PKA activities in female chickens. Protein kinase C, PKC, (**A**) and proteink kinase A, PKA, (**B**) activities in $1,25(OH)_2D_3$-treated cells as percent of vehicle controls are presented. Intestinal cells from 7-, 14-, 28-, and 58-week-old female chickens were isolated and treated with either 130 pM $1,25(OH)_2D_3$ or ethanol. For PKC cells were treated for 5 min and for PKA cells were treated for 7 min. Significant differences between age groups (small letters) were either obtained by an one-way ANOVA followed by Student's Newman-Keuls post-hoc test or by linear regression analysis. R^2 for coefficient of determination and $_{adj}R^2$ the adjusted coefficient of determination. Data are presented as mean ± SEM and $P<0.05$ for significance. For PKC n = 3-7 and for PKA n= 3 independent cell preparations for each age group tested.

as percent of controls, respectively. The changes in PKC activity were calculated as 150 ± 5.5, 120 ± 19, 100 ± 4.4, and 84± 4.1 % (n = 3-7), respectively, for 7-, 14-, 28-, and 58-week-old birds. When comparing $1,25(OH)_2D_3$ induction of PKC activity between the different ages, results from both a one-way ANOVA followed by Student's Newman-Keuls post-hoc test and linear regression analysis ($P<0.05$, F = 18, DF = 21, $R^2 = 0.47$ and $_{adj}R^2 = 0.45$) gave a significant ($P<0.05$) linear decrease in activity (Fig. 3.11A). Steroid mediated changes in PKA activity, expressed as percent of corresponding controls, were calculated to be 160 ± 30, 180 ± 42, 260 ± 40, and 320 ± 46 % (all, n = 3), respectively, for 7-, 14-, 28-, and 58-week-old birds. No significant differences were found when comparing $1,25(OH)_2D_3$-stimulated activity between the different age groups with a one-way ANOVA followed by Student's Newman-Keuls posthoc test, while a linear regression analysis ($P<0.05$, F = 11, DF = 11, $R^2 = 0.53$ and $_{adj}R^2 = 0.49$) on the same data showed a significant linear increase in the steroid-enhanced activity with ageing (Fig. 3.11B). Thus our results show an age related decrease in $1,25(OH)_2D_3$ stimulated PKC activity while the steroid-enhanced PKA activity increased with maturation.

Discussion

In the current work, age-related changes in $1,25(OH)_2D_3$-induced calcium transport were studied in female chickens ranging in age from pullets to egg-laying hens. Each age has a high demand for calcium, but for different reasons: young birds require substantial calcium for bone formation during growth, while mature hens require calcium for shell formation. However, the results indicate that the rapid, $1,25(OH)_2D_3$- mediated

increase in intestinal calcium transport declines with progressive age.

Basal intestinal calcium transport in immature (age 11 weeks), point-of-laying (age 17 weeks) and egg laying birds (age 25 weeks) has been reported to increase 2-fold between point of laying and egg laying birds, while the basal calcium transport was not significantly changed when comparing immature and point of laying birds (36). Thus, one interpretation of the combined data is that if calcium transport in egg laying birds is at, or close to, maximum, a further stimulation with $1,25(OH)_2D_3$ will not have as a great effect on the transport as a stimulation in younger birds having lower basal calcium absorption. Thus, our results show that young growing birds having a high requirement for calcium to produce bone respond more to $1,25(OH)_2D_3$ stimulation than older birds. The onset of egg laying and egg laying itself does not seem to have any effect on $1,25(OH)_2D_3$ induced calcium transport, even though the calcium needs are also high. The higher need for calcium in egg laying birds is most probably obtained by a higher basal intestinal calcium uptake in addition to ion uptake by the egg shell gland.

In order to further understand the age-related decline in the rapid, $1,25(OH)_2D_3$-mediated stimulation of intestinal calcium transport, we studied the binding properties of the nuclear VDR and the $1,25D_3$-MARRS bp. Analysis of $[^3H]1,25(OH)_2D_3$ binding to nVDR, could be described as hyperbolic function and Hill analysis of the data gave n_{app} close to 1 showing no significant differences between age groups. Thus, an absence of cooperative binding has been shown for nVDR. No changes in K_d were observed with increasing age, and all of the derived K_d values were slightly lower than circulating $1,25(OH)_2D_3$ levels, but still in the physiological range, since circulating $1,25(OH)_2D_3$ levels in female chicken have been reported to be 0.28 ± 0.06, and 0.051 ± 0.005 nM for

young and old chickens respectively (37). It has been reported earlier that the K_d of the nVDR is unaffected by age (26, 38), which agrees with the results presented in this study. Furthermore, while the rapid hormone stimulation of calcium transport declined with age the B_{max} increased with age for nVDR. A number of other studies have shown a decrease in both nVDR protein and mRNA levels in intestine with increasing age (22, 24, 26, 38), which have been proposed to be one factor causing the observed age-related decrease in $1,25(OH)_2D_3$ responsiveness. However, our results showed no correlation between changes in B_{max}, which is the maximal binding capacity of the receptor, indirectly indicating no correlation in number of unoccupied receptors and increasing age. In agreement with our result are those found in rat (21) and humans (23).

$[^3H]1,25(OH)_2D_3$ saturation binding curves for the $1,25D_3$-MARRS bp fitted a sigmoid function, and Hill analysis gave n_{app} significantly greater than 1. These results reveal a presence of allosteric interactions and cooperative binding. Positive cooperativity of $1,25(OH)_2D_3$ binding to a membrane receptor has been reported earlier in the rat osteosarcoma cell line UMR-106 and mouse derived osteoblast-like cells, MC-3T3-E1 (39) and in carp intestinal cells (40). The observed positively cooperative binding is not restricted to $1,25(OH)_2D_3$ binding to membrane receptors. The steroids, $24,25(OH)_2D_3$ (41), estradiol (42-44) and progesterone (45) have also been demonstrated to bind to plasma membranes with positive cooperativity. Our data however do not give any information on the nature of the cooperative receptor-hormone binding; that is, whether it is due to interactions of a number of receptor subunits or due to interaction with some other plamalemmal moiety. The K_d values for $1,25D_3$-MARRS bp were slightly higher than circulating $1,25(OH)_2D_3$ concentrations in pullets, but still in the physiological

range. A maturation associated increase in K_d was found for $1,25D_3$-MARRS bp, indicating a decreased affinity, which may explain the age-related decrease in intestinal responsiveness to $1,25(OH)_2D_3$. The maximal binding capacity or number of unoccupied binding sites, B_{max}, on the other hand significantly increased with maturation. SDS-PAGE followed by Western blot analyses also revealed an increase in number of $1,25(OH)_2D_3$ receptors with increasing age, supporting the results obtained in the saturation binding analyses. The increased number of binding sites may in part offset the decreased affinity to account for the diminished, but not abolished levels of rapidly stimulated calcium transport by hormone.

When comparing affinity and maximal binding capacity for nVDR and $1,25D_3$-MARRS bp the latter reveled both higher K_d and B_{max} for all ages tested. Thus, a difference in physiochemical properties between the two receptors has been shown giving proof for the existence of a membrane receptor different from nVDR. These results run counter to the argument that the nVDR must be responsible for membrane-initiated phenomena (46).

Besides receptor levels and affinity, it is, in the aging process, important to consider changes in cellular signal-transduction pathways, since these are related to steroid stimulated calcium transport. PKC is known to play a key role in regulation of cell growth, differentiation and ion channel modulation (47). In rat duodenum an age-related impairment of phospholipase C stimulation by $1,25(OH)_2D_3$, production of the second messengers IP_3 and DAG (28), and an altered hormone regulation of calcium transport through the PKC messenger system has been reported (27). The results obtained in this study demonstrate that $1,25(OH)_2D_3$ induced PKC activity decreased with age. This is in

agreement with earlier results (27), and may contribute to the observed age-related decrease in intestinal calcium transport in response to $1,25(OH)_2D_3$ stimulation. In addition to PKC, PKA has been proposed to stimulate intestinal calcium transport by activating voltage-gated calcium channels at the basal lateral membrane. This activation in turn leads to a transient increase in intracellular calcium which may activate exocytosis of calcium-containing vesicles (11), as well as calcium efflux by the calcium pump and the Na^+/Ca^{2+} exchanger, resulting in a net increase in duodenal calcium transport (6-8). In the present work, an induction of PKA activity by hormone was found in all age groups tested. In contrast to Massheimer *et al.* (25), who have reported an age-related fall in the stimulation of cAMP/protein kinase A-dependent calcium uptake by $1,25(OH)_2D_3$ in rat duodenum, our study showed a linear increase in PKA activity with maturation. Thus it seems likely that the PKA activity does not have the proposed effect on the intestinal cell calcium pumps. The increase in PKA activity with age cannot be explained at this point and needs further investigation.

In conclusion our results show that young growing birds having high calcium requirements respond more robustly to $1,25(OH)_2D_3$ stimulation than older birds. The decreased responsiveness can be explained both by a decreased affinity of $1,25D_3$-MARRS bp for $1,25(OH)_2D_3$, and decreased PKC activity. The onset of egg laying and egg laying itself does not seem to have any effect on the rapid $1,25(OH)_2D_3$ induced intestinal calcium uptake even though the calcium needs are again high. Further, this study gives proof for the existence of a membrane associated $1,25(OH)_2D_3$ receptor having different physiochemical properties from the classical nVDR.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

$1,25(OH)_2D_3$ is the principal regulator of intestinal calcium absorption. Thus, changes in the vitamin D endocrine system with age are implicated in the pathogenesis of calcium malabsorption. Aging may affect the vitamin D endocrine system in different ways. A low dietary intake of vitamin D (1), inadequate exposure to sunlight (2), and a progressive decline in renal function with age, resulting in a decreased 25-hydroxyvitamin D 1α–hydroxylase activity (3, 4), may lead to decreased levels of $1,25(OH)_2D_3$. The decreased levels of $1,25(OH)_2D_3$ in turn may cause a reduced intestinal calcium uptake. Furthermore, the capacity of $1.25(OH)_2D_3$ to stimulate calcium absorption also declines with age (5, 6), but the biochemical mechanisms responsible for this decrease in intestinal function are not fully understood.

The studies in this dissertation have addressed the biochemical mechanisms responsible for the age-associated decrease in the capacity of $1,25(OH)_2D_3$ to stimulate calcium absorption in both male and female chickens.

Age-related changes in $1,25(OH)_2D_3$-induced intestinal calcium uptake were studied in both male and female chickens of ages 7, 14, 28, and 58 weeks. Both genders and all ages tested showed an increase in intestinal calcium uptake after treatment with 130 pM $1,25(OH)_2D_3$ (Fig. 2.2, 3.2 and Table A.1). The responsiveness to $1,25(OH)_2D_3$ declined with increasing age in both male and female birds, the decrease being highest between 7 and 14 weeks. A negative correlation between changes in intestinal calcium

uptake and age was found in both male and female chicken duodenum (Fig. 2.3, 3.3, Table A.6, and A.7). The change in $1,25(OH)_2D_3$ induced intestinal calcium uptake was also negatively correlated to weight in both male and female chickens (Table A.6 and A.7). These results can be explained by the fact that growing animals require large amounts of dietary calcium to build bones, while the adult animals would have less need of rapidly stimulated intestinal transport. Consequently, the rapid effect of $1,25(OH)_2D_3$ on intestinal calcium transport, mediated through the $1,25D_3$-MARRS bp, would be expected to decrease. This is true for male birds. In contrast, adult hens continue to require large amounts of calcium to produce eggshells, which might be reflected by a continued responsiveness to $1,25(OH)_2D_3$, as judged by rapid stimulation of calcium transport. However this was not the case. The onset of egg laying and egg laying itself resulted in a reduced responsiveness to $1,25(OH)_2D_3$ compared to non egg laying birds, even though the calcium needs in mature birds are high. One explanation for this observation may be that if the intestinal calcium uptake in female birds is close to maximum, a further stimulation with $1,25(OH)_2D_3$ would not have as great effect on the transport as a stimulation in animals having lower basal calcium uptake. Thus, egg laying hens having high basal intestinal calcium uptake would respond less to $1,25(OH)_2D_3$ than young chickens having lower basal calcium uptake. Another explanation may be that the decreased demand for calcium for growth, together with an increased calcium uptake by the egg shell gland are factors resulting in the observed decrease in $1,25(OH)_2D_3$ induced intestinal calcium transport seen in adult female birds.

Male and female birds of the same ages did not show any differences in $1,25(OH)_2D_3$ induced intestinal calcium transport after 40 minutes vascular perfusion

with the steroid (Table A.1).

At the cellular level, $1,25(OH)_2D_3$ is known to exert its effects on target cells by two mechanisms, a slow and a rapid mechanism. In the slow mechanism, $1,25(OH)_2D_3$ mediates its effects by binding to nVDR, while the rapid mechanism is mediated by membrane associated $1,25D_3$-MARRS bp. Saturation binding analysis of $1,25(OH)_2D_3$ to nVDR and $1,25D_3$-MARRS bp were performed to investigate which hormone-mediated pathway is affected by increasing age, and may contribute to the reduced hormone responsiveness observed in intestinal calcium transport.

Results obtained from $[^3H]1,25(OH)_2D_3$ saturation binding analysis to nVDR followed by Hill analysis of the data obtained, showed an absence of cooperative binding in both male and female-birds of ages 7, 14, 28, and 58 weeks. No changes in K_d were observed with increasing age (Table A.6 and A.7), which is in agreement with earlier reported results (7, 8). This lack of change in affinity makes it unlikely that nVDR is responsible for mediating the rapid stimulation of calcium transport that declines with age. Furthermore, a comparison of affinity between genders for the different age groups, showed a significant difference between 7-week male and 7-week female birds, where male birds had a lower affinity. No significant differences in K_d were observed when comparing the male and female birds of 14, 28, and 58 weeks of age (Table A.2). If K_d values for male and female birds were calculated by combining all data for the different ages, a comparison did not show any significant difference in K_d between the genders (data not shown).

A number of studies have shown a decrease in both nVDR protein and mRNA levels in intestine with increasing age (7-10). This has been proposed to be one factor

causing the observed age-related decrease in intestinal $1,25(OH)_2D_3$ responsiveness. However, our results showed no correlation between age or weight and changes in B_{max} for male and female birds (Table A.6 and A.7), indicating no correlation to changes in the maximal binding capacity of the receptor, and thus no correlation to changes in number of unoccupied receptors. In agreement with our results, however, are those found in rat and humans by Wood *et al.* (6) and Kinyamu *et al.* (11), respectively. A comparison of B_{max} for the different ages between genders, showed a significant difference only in 7-week-old birds (Table A.2). Thus, both K_d and B_{max} were found to be significantly lower in 7-week-old female birds compared to male birds of the same age. Accordingly, nVDR in female birds have higher affinity for the steroid than nVDR in male birds, while the number of unoccupied binding sites are less in 7-week-old female birds than in male birds of the same age. An explanation for this finding is difficult to give and needs further investigation.

$1,25(OH)_2D_3$ is also known to exert its effects on target cells by a rapid mechanism. The presence of rapid action of $1,25(OH)_2D_3$ has been known for over two decades and a number of hypotheses have been proposed to explain this event. It has been proposed that: (i) the nuclear nVDR is responsible for the observed rapid actions, (ii) $1,25(OH)_2D_3$ itself has the ability to directly activate signal transduction pathways and (iii) a new, membrane bound $1,25(OH)_2D_3$ vitamin D_3 receptor exists and functions like a peptide hormone receptor. Today a great number of reports support the last hypothesis saying that a new receptor different from the nVDR, $1,25D_3$-MARRS bp, is responsible for the rapid actions of $1,25(OH)_2D_3$. Therefore, the work in this dissertation examined age-related changes in expression and physiochemical properties of $1,25D_3$-MARRS bp

in male and female chickens.

[^{3}H]1,25(OH)$_2$D$_3$ saturation binding analyses of 1,25D$_3$-MARRS bp followed by Hill analysis revealed the presence of allosteric interactions and positive, cooperative binding in both male and female birds of ages 7, 14, 28, and 58 weeks. Positive cooperativity of 1,25(OH)$_2$D$_3$ binding to a membrane receptors has previously been reported in both osteoblastic cells from rat and intestinal cells from carp (12, 13) and it has been shown that estradiol, progesterone and 24,25(OH)$_2$D$_3$ bind plasma membranes with positive cooperativety (14-18). Thus, these results agree with earlier findings for membrane associated steroid receptors. These data however do not give any information on the nature of the cooperative receptor-hormone binding; that is, whether it is due to interactions of a number of receptor subunits or due to interaction with some other component.

An age-related increase in K_d was found for 1,25D$_3$-MARRS bp in both male and female birds, indicating a decreased affinity (Fig. 2.6 and 3.6). This diminished affinity has been suggested to explain the age-related decrease in intestinal responsiveness to 1,25(OH)$_2$D$_3$. Regression analyses on male and female birds showed significant linear relation between weight and K_d (Table A.6 and A.7). Analysis on male birds also showed a linear increase in K_d with age, while the age was not significantly correlated to K_d in female birds (Table A.6 and A.7). Because an increase in weight during growth is normally dependent on age, these data indirectly indicate that K_d for 1,25D$_3$-MARRS bp increases with increasing age during growth in female chickens. To investigate whether the observed decrease in intestinal calcium uptake with age is due to a decreased affinity of 1,25D$_3$-MARRS bp, correlation analyses between changes in K_d and calcium uptake

after 40 min perfusion with 130 pM $1,25(OH)_2D_3$ were performed. The results obtained showed a negative correlation between intestinal calcium uptake and K_d in female chickens, while no correlation was obtained for male birds (Table A.8). Thus, based on these results the decrease in $1,25D_3$-MARRS bp affinity can explain the decreased intestinal calcium uptake as a response to $1,25(OH)_2D_3$ in female birds. A comparison of K_d for $1,25D_3$-MARRS bp between male and female birds of the same age showed a significantly higher affinity in male 28 week old birds compared to female birds of the same age. No gender-associated differences in K_d were found for 7-, 14-, and 58-week-old birds (Table A.2).

The maximal binding capacity or number of unoccupied binding sites, B_{max}, significantly increased with maturation in female birds. Furthermore, SDS-PAGE followed by Western blot analyses made on female chickens revealed an increase in the number of $1,25D_3$-MARRS bp receptors between 28 and 58 weeks of age, suggesting that the increase in number of unoccupied binding sites may be, at least in part, due to an increased number of receptors (Fig. 3.6 and 3.8). A correlation analysis of the data on the other hand did not show any correlation between B_{max} and expression of $1,25D_3$-MARRS bp (Table A.8). Male birds showed an increase in B_{max} between 7 and 14 weeks, which decreased at 28 weeks, while Western analyses revealed a linear decrease in receptor expression (Fig. 2.6, 2.8, and Table A.6). It should be noted that B_{max} in 7-week-old male birds may be underestimated in the current work, since previously, a B_{max} of 250 fmol/mg protein was reported (19) in young birds, while the B_{max} in this work was determined to be 189 ± 18.2 fmol/mg protein. Furthermore, in the binding analyses of 58-week-old male birds saturation was not obtained within the range of concentrations tested, resulting

in an invalid B_{max} value.

When comparing B_{max} for $1,25D_3$-MARRS bp between male and female birds of the same age, 14- and 28-week-old female birds showed significantly higher number of unoccupied binding sites compared to male birds of the same age (Table A.3). Since B_{max} in male birds may be underestimated in the 7-week age group, and no saturation was obtained for $1,25D_3$-MARRS bp at 58 weeks, no comparisons of B_{max} between male and female birds were performed for these ages.

A comparison of expression of $1,25D_3$-MARRS bp between male and female birds of the same age revealed a higher expression of the receptor in 58-week-old female chickens compared to male chickens of the same age. No differences in expression were found when comparing 7-, 14-, and 28-week-old male birds with female chickens of the corresponding ages (Table A.4).

In conclusion, both male and female birds showed a decrease in $1,25D_3$-MARRS bp affinity for $1,25(OH)_2D_3$, which was correlated to changes in weight (growth). In female birds the decreased affinity was also significantly correlated to changes in $1,25(OH)_2D_3$ induced calcium uptake, and can thus explain the observed decrease in intestinal responsiveness to $1,25(OH)_2D_3$. Changes in K_d for $1,25D_3$-MARRS bp with increasing age did not show any correlation to changes in intestinal calcium uptake in response to $1,25(OH)_2D_3$ in male birds. B_{max} depends on a number of factors, such as concentration of circulating $1,25(OH)_2D_3$, number of receptors and presence of other hormones that are able to interact with the vitamin D system. Thus, it is difficult to directly correlate changes in B_{max} to changes in $1,25(OH)_2D_3$ induced intestinal calcium uptake, without considering other interacting factors. Future studies are necessary to

address the regulating factors responsible for the observed changes in B_{max} for $1,25D_3$-MARRS bp.

The presence or absence of signal transduction pathways is related to steroid-stimulated calcium transport, thus making it another important point to consider in the aging process aside from receptor levels and affinity. The results obtained in the current study, demonstrated that $1,25(OH)_2D_3$ induced less PKC activity with age in both male and female chickens, although at different times (Fig. 2.11 and 3.11), is in agreement with earlier reports (20, 21). In both male and female birds the change in $1,25(OH)_2D_3$ induced PKC activity was negatively correlated to both age and weight (Table A.6 and A.7). Furthermore, changes in $1,25(OH)_2D_3$ induced PKC activities were correlated to changes in steroid induced intestinal calcium uptake in female birds, while male birds did not show any significant correlation. In both male and female birds changes in induced PKC activity showed a negative correlation to changes in K_d of $1,25D_3$-MARRS bp with age (Table A.8). Thus, in female birds the changes in K_d of $1,25D_3$-MARRS bp explains the reduced induction in PKC activity, which in turn is correlated to age related changes in $1,25(OH)_2D_3$ induced intestinal calcium uptake. In male birds changes in K_d of $1,25D_3$-MARRS bp are correlated to changes in $1,25(OH)_2D_3$ induced PKC activity, while no direct correlation was found neither between changes in K_d or PKC and $1,25(OH)_2D_3$ induced intestinal calcium uptake. Moreover, the current work demonstrates a decrease in basal PKC activity in male chickens at age 14 weeks, while female chickens did not show any significant changes in basal PKC activities with age (Fig. 2.10 and 3.10). Earlier studies by Balogh *et al.* (20) on rat duodenum have reported changes in basal PKC activity with senescence, which contrary to our findings, showed increased activity as a

function of age. At present, we speculate that the differences are species related. A comparison of $1,25(OH)_2D_3$ induced PKC activity, expressed as percent of control, between male and female chickens, showed a higher activity in 28- and 58-week-old male birds compared to female birds of the corresponding ages (Table A.5). No significant differences in PKC activity induction were observed when comparing 7- and 14-week-old male chickens with female birds of corresponding ages (Table A.5).

In the current work, $1,25(OH)_2D_3$ treatment of isolated intestinal epithelial cells resulted in an induction of PKA in both male and female chickens in all age groups tested. The hormonal stimulation was significant for 7- and 58-week-old male birds, and for 28- and 58-week-old female birds (Fig. 2.10 and 3.10). Furthermore, our study showed an increase in stimulated PKA activity with age in both male and female birds (Fig. 2.11, 3.11 and Table A.6 and A.7). No differences were observed when comparing stimulated PKA activity between male and female chickens within the different age groups.

In conclusion, the unimpaired PKA stimulation by the seco-steroid apparently allows the diminished, but not abolished enhancement of calcium transport in mature animals, whereas the contribution of the PKC pathway in young animals leads to a more robust absorption.

A summary of second messenger systems affected by $1,25D_3$-MARSS bp is illustrated in Fig. 4.1. As shown in this dissertation, $1,25D_3$-MARRS bp is an allosteric protein, which binds to its ligand with positive cooperativity. Hence, an effector protein is in involved in the binding. Whether the cooperative binding is mediated by interactions between the same or different subunits of the receptor, or whether a separate protein

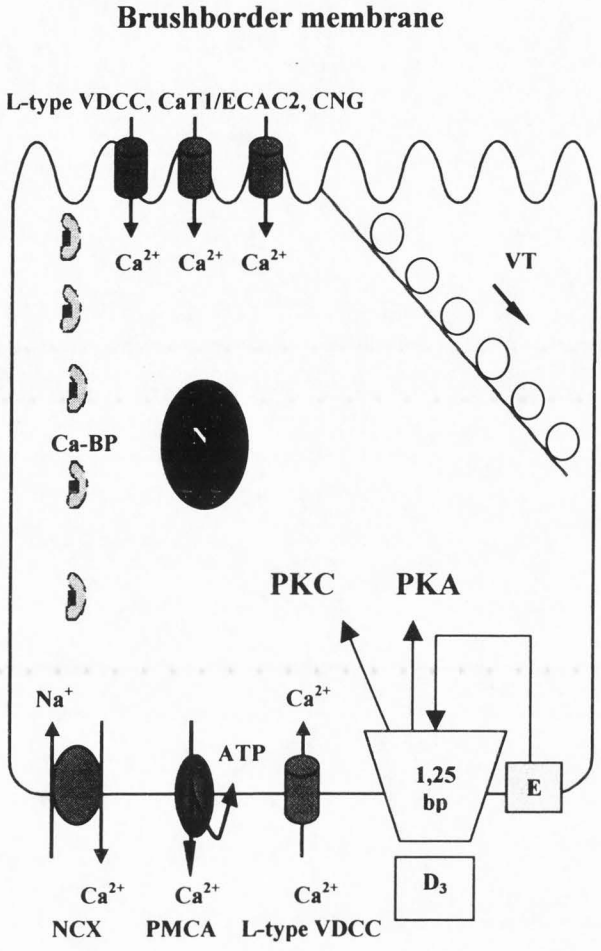

Fig. 4.1. Rapid actions of $1,25(OH)_2D_3$ on intracellular second messenger systems in the enterocyte. Calcium is takein to the cell at the apical membrane by L-type voltage directed calcium channels (L-type VDCC), calcium transporter 1/epitheliat calcium channel 2 (CaT1/ECAC2) and cyclic nucleotide gated channels (CNG). Inside the cell calcium can either be transported bound to calcium binding proteins (Ca-BP) in the cytoplasm and in the vesiculer transporters (VT). At the basal lateral membrane Na/Ca^{2+}-exchangers (NCX) or plasma membrane Ca^{2+}-ATPases (PMCA), and vesicular exocytosis transport calcium out from the cell over the basal lateral membrane. Transport over the enterocyte can be regulated by $1,25(OH)_2D_3$ (D_3). As the hormone binds to $1,25D_3$-MARRS bp (1,25 bp) it can stimulate production of protein kinase C (PKC) and protein kinase A (PKA). The induced second messenger systems may regulate both protein transcription in the nucleus (N) or directly affect rapid intestinal calcium uptake. $1,25D_3$-MARRS bp has been shown to be an allosteric protein and thus may be affected by an effector protein unit (E).

mediates the cooperativity, is not known at present. The affinity of $1,25D_3$-MARRS bp for its ligand decreases with age. This could be due to the nature of the receptor, the nature of the effector, or a combination of both. What causes the decrease is not known.

As $1,25(OH)_2D_3$ binds to $1,25D_3$-MARRS bp it has the potential to activate both PKC and PKA. In both male and female birds the induced PKC activity is reduced with increasing age. The decreased activation of PKC can be explained by an age-related decrease in $1,25D_3$-MARRS bp affinity for $1,25(OH)_2D_3$ with increasing age. In female birds, a direct correlation between changes in $1,25(OH)_2D_3$ induced intestinal calcium uptake and reduced induction in PKC activity was found. Steroid induced PKA activity increased with age in both male and female birds. Since $1,25(OH)_2D_3$ induced intestinal calcium uptake decreases with age while the induced PKA activity increases we may conclude that changes in PKA activity are not representative for the age related decline in calcium transport. PKC and intracellular calcium can affect rapid calcium transport over the enterocyte by a number of mechanisms. The effect can be exerted on calcium channels, which can be either activated or inhibited, thus regulating both calcium uptake into the cell, over the brushborder membrane, and calcium transport out from the cell, over the basal lateral membrane. Furthermore, changes in both protein kinase activity and intracellular calcium levels have the potential to regulate the vesicular transport, thus affecting the rapid vesicular transport over the enterocyte. How a decrease in PKC activity and a parallel decreased intracellular signaling by calcium affects transcription, calcium channel activities, and vesicular transport is not known at present, and needs further investigation.

Conclusions

1. There is an age related decrease in intestinal calcium uptake in response to exogenous $1,25(OH)_2D_3$ in duodena of both male and female chickens.
2. In female chickens, the decreased responsiveness to exogenous $1,25(OH)_2D_3$ can be explained by a decreased affinity of $1,25D_3$-MARRS bp for the steroid.
3. Male birds show a reduced expression of $1,25D_3$-MARRS bp with increasing age, which in part may explain the observed decrease in responsiveness to exogenous $1,25(OH)_2D_3$.
4. Female birds showed an increased expression of $1,25D_3$-MARRS bp at the age 58 weeks.
5. No correlation between age and changes in the affinity and number of binding sites for nVDR were observed in male and female chickens, indicating that this receptor is of minor importance in the age related decrease in intestinal calcium uptake in response to exogenous $1,25(OH)_2D_3$.
6. Saturation binding analyses of $[^3H]1,25(OH)_2D_3$ to nuclear fraction nVDR indicate an absence of cooperative binding, while saturation binding analyses of $[^3H]1,25(OH)_2D_3$ to $1,25D_3$-MARRS bp in BLM showed the presence of allosteric interaction and positive cooperative binding. These results give further proof for the existence of a membrane associated $1,25(OH)_2D_3$ receptor having different physiochemical properties from the classical nVDR.
7. $1,25(OH)_2D_3$-induced PKC activity decreases with age in both male and female chickens. In both genders the decrease in PKC activity was correlated to a

decreased $1,25D_3$-MARRS bp affinity for $1,25(OH)_2D_3$. In female birds, the age-related changes in $1,25(OH)_2D_3$ induced PKC activity was positively correlated to changes in steroid induced intestinal calcium uptake.

8. Both male and female chickens showed an increase in stimulated PKA activity as a function of age. The significance of an increased PKA activity in the aging process needs further investigation.

9. Finally, a physiological importance of the membrane receptor in senescence related changes in responsiveness to $1,25(OH)_2D_3$ in intestine has been demonstrated.

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APPENDIXES

Appendix A. Comparison of data between male and female chickens, and correlation analyses

Table A.1

Rapid stimulation of calcium transport in male and female chickens

	^{45}Ca in venous effluent at T = 40 min (Treatment/Av Basal)	
Age (weeks)	Males	Females
7	2.6 ± 0.4	3.0 ± 0.5
14	1.9 ± 0.4	1.7 ± 0.2
28	1.8 ± 0.4	1.7 ± 0.4
58	1.5 ± 0.2	1.4 ± 0.2

Data presented in Fig. 2.2, 2.3, 3.2, and 3.3 were used for comparison of $1,25(OH)_2D_3$ induced changes in calcium transport between male and female chickens of same age. The change in calcium transport was determined by a one-way ANOVA followed by Student's Newman-Keuls post-hoc test. No differences in ^{45}Ca in venous effluent at T = 40 min between male and female birds of the same ages were observed. Data are presented as mean ± SEM (n=3-5).

Table A.2

Receptor affinities in male and female chickens

	Males		Females	
	K_d (pM)		K_d (pM)	
Age (weeks)	nVDR	1,25D_3-MARRS bp	nVDR	1,25D_3-MARRS bp
7	350 ± 120	590 ± 130	80 ± 20*	830 ± 160
14	380 ± 170	1030 ± 100	200 ± 60	1300 ± 190
28	290 ± 180	990 ± 70	60 ± 40	1600 ± 210*
58	300 ± 110	2700 ± 840	110 ± 40	1500 ± 220

Data presented in Figs 2.4, 2.6, 3.4, and 3.6 were used for comparison of changes in receptor affinities between male and female chickens of the same ages. Statistical comparisons of K_d values between male and female birds were made for each receptor within each age group. Statistical comparisons were determined with Student's t-test. Data are presented as mean ± SEM for three independent preparations in each age group. *$P<0.05$ differences are relative to values calulated for the corresponding receptor preparation from male birds.

Table A.3

Number of unoccupied receptor binding sites in male and female chickens

	Males		Females	
	B_{max} (fmol/mg protein)		B_{max} (fmol/mg protein)	
Age (weeks)	nVDR	1,25D_3-MARRS bp	nVDR	1,25D_3-MARRS bp
7	31 ± 3.9	190 ± 18	19 ± 1.0*	210 ± 21
14	36 ± 5.2	250 ± 13	30 ± 2.6	330 ± 30 *
28	31 ± 5.1	190 ± 11	26 ± 3.0	290 ± 24 *
58	23 ± 2.6	640 ± 160	29 ± 2.5	460 ± 44

Data presented in Figs 2.4, 2.6, 3.4, and 3.6 were used for comparison of changes in number of unoccupied receptor binding sites between male and female chickens of the same age. Statistical comparisons of B_{max} values between male and female birds were made for each receptor within each age group. Statistical comparisons were determined by Student's t-test. Data are presented as mean ± SEM for three independent preparations in each age group. *$P<0.05$ differences are relative to values calulated for the corresponding receptor preparation from male birds.

Table A.4

Expression of $1,25D_3$-MARRS bp in BLM

	Males	Females
Age (weeks)	Relative Density	Relative Density
7	1.0 ± 0.1	1.0 ± 0.1
14	0.6 ± 0.1	0.9 ± 0.1
28	0.8 ± 0.1	0.9 ± 0.1
58	0.7 ± 0.1	1.2 ± 0.1*

Data presented in Figs 2.8 and 3.8 were used for comparison of changes in expression of $1,25D_3$-MARRS bp between male and female chickens of the same age. Significant differences were obtained by Student's Newman-Keuls post-hoc test. Data are presented as mean ± SEM, n = 8-14 from at least 3 separate runs. *$P<0.05$, relative to corresponding values in preparations from male birds.

Table A.5

Changes in $1,25(OH)_2D_3$ induced PKC and PKA activities in male and female chickens

	Males		Females	
	Changes in activity (% of control)		Changes in activity (% of control)	
Age (weeks)	PKC	PKA	PKC	PKA
7	160 ± 14	200 ± 21	150 ± 5.5	160 ± 30
14	100 ± 21	160 ± 15	120 ± 19	180 ± 42
28	140 ± 17	200 ± 42	100 ± 4.4*	260 ± 40
58	34 ± 8.7	270 ± 10	84 ± 4.1*	320 ± 46

Data presented in Figs 2.11 and 3.11 were used for comparison of changes in PKC and PKA activities between male and female birds of the same age. Significant differences were obtained by a one-way ANOVA followed by Student's Newman-Keuls post-hoc test. Data are presented as mean ± SEM (n = 3-14). *$P<0.05$, relative to corresponding values in preparations from male birds.

Table A.6

Regression analyses; male chickens

	Age		Weight	
	P-value	R^2	*P*-value	R^2
Ca (40 min)	0.04*	0.25	0.08	0.21
B_{max} (nVDR)	0.14	0.74	0.17	0.69
K_d (nVDR)	0.30	0.49	0.37	0.39
B_{max} ($1,25D_3$-MARRS bp)	0.10	0.81	0.07	0.87
K_d ($1,25D_3$-MARRS bp)	0.04*	0.92	0.02*	0.96
Density ($1,25D_3$-MARRS bp)	0.04*	0.11	0.04*	0.10
PKC	0.0004*	0.40	0.0002*	0.44
PKA	0.03*	0.28	0.04*	0.25

Regression analyses were used in comparing age or weight to changes in intestinal calcium transport determined after 40 min of perfusion, B_{max} and K_d for both nVDR and $1,25D_3$-MARRS bp the expression of $1,25D_3$-MARRS bp, PKC and PKA activities expressed as percent of control, respectively. The data used were earlier presented in table A.1-A.5. The coefficient of determination and the *P*-values are presented in the table. $P<0.05$ for significance which is indicated by *.

Table A.7

Regression analyses; female chickens

	Age		Weight	
	P-value	R^2	*P*-value	R^2
Ca (40 min)	0.02*	0.27	0.01*	0.29
B_{max} (nVDR)	0.48	0.27	0.31	0.47
K_d (nVDR)	0.85	0.02	0.89	0.01
B_{max} ($1,25D_3$-MARRS bp)	0.10	0.81	0.18	0.67
K_d ($1,25D_3$-MARRS bp)	0.27	0.53	0.04*	0.92
Density ($1,25D_3$-MARRS bp)	0.49	0.26	0.75	0.06
PKC	0.002*	0.40	0.0005*	0.47
PKA	0.01*	0.52	0.01*	0.51

Regression analyses were used in comparing age or weight to changes in intestinal calcium transport determined after 40 min of perfusion, B_{max} and K_d for both the nVDR and $1,25D_3$-MARRS bp, the expression of $1,25D_3$-MARRS bp, PKC and PKA activities expressed as percent of control. The data used were earlier presented in table A.1-A.5. The coefficient of determination and the *P*-values are presented in the table. $P<0.05$ for significance which is indicated by *.

Table A.8

Pearson correlation analyses; male and female chickens

		($1,25D_3$-MARRS bp)			
	Ca (40 min)	B_{max}	K_d	PKC	PKA
MALES					
Ca (40 min)	-----	-0.68	-0.78	0.80	-0.46
B_{max} ($1,25D_3$-MARRS bp)	-----	-----	(0.98*)	-0.98*	0.85
K_d ($1,25D_3$-MARRS bp)	-----	-----	-----	-0.98*	0.85
FEMALES					
Ca (40 min)	-----	-0.84	-0.93*	0.92*	-0.74
B_{max} ($1,25D_3$-MARRS bp)	-----	-----	(0.66)	-0.86	-0.81
K_d ($1,25D_3$-MARRS bp)	-----	-----	-----	-0.93*	0.79

Pearson correlation analyses were used to compare two dependent parameters. Analyses were made on intestinal calcium transport determined after 40 min of perfusion, B_{max} and K_d for the $1,25D_3$-MARRS bp, PKC, and PKA activities expressed as percent of control. The data used were earlier presented in table A.1-A.3 and A.V. Pearson correlation coefficients are presented in the table. $P<0.05$ for significance which is indicated by *. Analyses that are not of physiological relevance are in brackets.

Appendix B. Protocols for determination of PKC and PKA activities, and protein levels

Protein kinase C assay (GIBCOBRL LIFE TECNOLOGIES)

1. Homogenize cell pellets (15 strokes, glass-teflon homogenizer) in extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 % Triton X-100, 25 μg/ml each aprotinin and leupeptin).
2. Incubate on ice 30 min and centrifuge 14 000 rpm 2 min. Analyze supernatant for PKC activity.
3. Incubate extract (10 μg protein/tube) at room temp 20 min in presence of 5 μl activator (100 mM phorbol 12-myristate 13 acetate [PMA], 2.8 mg/ml phosphatidyl serine, Triton X-100 micelles) or 10 μl inhibitor (100 μM PKC(19-36), 20 mM Tris pH 7.5).
4. Add [γ-^{32}P]ATP (20-25 μCi/ml) + substrate (250 μM acetylated myelin basic protein (Ac-MBP(4-14)), 100 μM ATP, 5 mM $CaCl_2$, 100 mM $MgCl_2$, 20 mM Tris pH 7.5) and mix. Incubate 5 min at 30 °C.
5. Spot 25 μl onto phosphocellulose discs.
6. Wash discs 2 x 5 min with 1% (v/v) phosphoric acid followed by 2 x 5 min washes in distilled water.
7. Transfer discs into scintillation vials, add scintillation fluid and assess incorporated ^{32}P.
8. Calculate specific PKC activity:

   Specific PKC activity = Activity without inhibitor-Activity in presence of inhibitor.

Protein kinase A assay (GIBCOBRL LIFE TECNOLOGIES)

1. Homogenize cell pellets (15 strokes, glass-teflon homogenizer) in extraction buffer (5 mM EDTA, 50 mM Tris, pH 7.5) and centrifuge at 14 000 x g 2 min. Analyze the supernatant for PKA activity.
2. Incubate extract (5 μg protein/tube) at room temp 20 min in presence of activator (40 μM cAMP, 50 mM Tris pH 7.5) or protein kinase inhibitor (4 μM PKI(6-22) amide, 50 mM Tris pH 7.5) only, in the presence of both activator and inhibitor, and in the absence of both.
3. Add [γ-^{32}P]ATP (20-25 μCi/ml) + substrate (200 μM Kemptide, 400 μM ATP, 40 mM $MgCl_2$, 1 mg/ml BSA, 50 mM Tris pH 7.5) and mix. Incubate 5 min at 30 °C.
4. Spot 20 μl onto phosphocellulose discs.
5. Wash the discs 2 x 5 min with 1% (v/v) phosphoric acid followed by 2 x 5 min washes in distilled water.
6. Transfer the discs into scintillation vials, add scintillation fluid and assess incorporated ^{32}P.7. Calculate the PKA activity:Amount activated PKA = Activity in absence of both activator and inhibitor - Activity in presence of inhibitor only.Total PKA activity = Activity in presence of activator only – Activity in presence of both activator and inhibitor

   % Activated PKA in cells = 100 (Amount activated PKA/Total PKA activity)Protein Assay (BIO-RAD)

1. Reconstitute the standard. To lyophilized bovine gamma globulin standard add 20 ml deionized water and mix until dissolved. If the standard will not used within

60 days, it should be aliquoted and frozen at –20 °C.

2. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water. Filter through Whatman #1 filter to remove particles. This diluted reagent may be used for approximately 2 weeks if kept at room temperature.
3. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for bovine gamma globulin is 0.2 to 1.5 mg/ml.
4. Pipet 100 ml of each standard and sample solution into a clean dry test tube. Protein solutions are normally assayed in duplicate or triplicate.
5. Add 5.0 ml of diluent dye reagent to each tube and vortex.
6. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
7. Measure absorbance 595 nm.
8. Make a standard curve and determine protein levels in the samples.

CURRICULUM VITAE

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Personal Data

Born in Gothenburg, Oct 10, 1967, Swedish citizen. Married to Dennis Larsson. One child, Daniel, born 1999.

Education and appointments

1983-87: Higher grammar school (Aschebergs Gymnasiet, Göteborg, Sweden). Graduated with a Major in chemical engineering.

1989-94: Undergraduate studies at Göteborg University, Sweden. Graduated with a Master of Science degree in Chemistry.

1994-99: Research work at the department of Oral Biochemistry, Göteborg University, Sweden.

1995-99: 36 hours of formal lectures in Biochemistry/Histology for dental hygienist curriculum, Göteborg University, Sweden.

1996-97: 4 hours of formal lectures and demonstrations in Oral Biology for dentist curriculum, Göteborg University, Sweden.

1996-99: 152 hours laboratory exercises Oral Biology for dentist curriculum, Göteborg University, Sweden.

2000-01: Maternity-leave.

2001-02: PhD student at the department of Nutrition and Food Sciences, Utah State University.

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Resarch experience

1993-95: Research on intracellular enzymes involved in the mineralization of dentin in rat odontoblasts. Department of Oral Biochemistry, Göteborg

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1996: Research on the endocrine regulation of calcium and phosphate transport in odontoblasts. Department of Oral Biochemistry, Göteborg University.

1997-99: Research studies on gastocalcin, a novel calciotropic and/or osteotropic hormone. Department of Oral Biochemistry, Göteborg University.

2001-02: PhD studies on the physiological importance of 1,25-dihydroxyvitamin D_3 non-nuclear receptors in male and female chicken as a function of growth and maturation, Utah State University.

Congress participation

1993: 2nd NOF/CED Joint Meeting, Kolding, Denmark.

1994: 77th Annual Meeting of NOF; Göteborg, Sweden.

1995: 5th International Conference on the Chemistry and Biology of Mineralized Tissues, Kohler, WI, USA.

1997: FEBS Advanced Course: Single Cell Techniques in Signal Transduction Research, Leiden/Amsterdam The Netherlands.

1997: The 80th Annual Meeting of NOF; 1997, Reykjavik, Iceland.

1998: 6th International Conference on the Chemistry and Biology of Mineralized Tissues, Vitell, France.

2001: 23rd Annual Meeting of the American Society for Bone and Mineral Research, Phoenix, Arizona, USA.

Publications

1. Larsson B, Linde A, Dahlin S 1996 Purification and characterization of alkaline phosphatase from rat incisor odontoblasts. Conn Tiss Res 35:243
2. Larsson B, Lundgren T, Linde A 1998 Voltage-gated Ca^{2+} channels in rat incisor odontoblasts. J Dent Res 77:1339
3. Larsson D, Larsson B, Lundgren T, Sundell K 1999 The Effect of pH and Temperature on the Dissociation Constant for Fura-2 and Their Effects on $[Ca^{2+}]_i$ in Enterocytes from a Poikilothermic Animal, Atlantic Cod (*Gadus morhua*). Anal Biochem 273:60-65

4. Larsson B, Gritli-Linde A, Norlén P, Lindström E, Håkanson R, Linde A 2001 Extracts of ECL-cell granules/vesicles and isolated ECL cells from rat oxyntic mucosa evoke a Ca^{2+} second messenger response in osteoblastic cells. Regul Pept 97:153-161
5. Larsson B, Nemere I 2001 Correlation of growth and maturation in roosters with magnitude of non-genomic responses to $1,25(OH)_2D_3$. J Bone Miner Res 16:S308
6. Larsson D, Axnes L, Björnsson B Th, Larsson B, Lundgren T, Sundell K 2002 Antagonistic effects of 24R,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 on L-type Ca^{2+} channels and Na^+/Ca^{2+} exchange in enterocytes from Atlantic cod (*Gardus Morhua*). J Mol Endocrinol 28:53-68
7. Larsson B, Norlén P, Lindström E, Zhao D, Håkanson R, Linde A 2002 Effects of ECL cell extracts and granule/vesicle-enriched fractions from rat oxyntic mucosa on cAMP and IP_3 in rat osteoblast-like cells. Regul Pept 106:13-18
8. Larsson B, Nemere I 2002 Effect of growth and maturation on membrane-initiated actions of 1,25-dihydroxyvitamin D_3. I. Calcium, receptor kinetics, and signal transduction in intestine of male chickens. Endocrinology (Submitted)
9. Larsson B, Nemere I 2002 Effect of growth and maturation on membrane-initiated actions of 1,25-dihydroxyvitamin D_3. II. Calcium transport, receptor kinetics, and signal transduction in intestine of female chickens. (Manuscript)

Review

1. Larsson D, Sundell K, Larsson B, Nemere I 2002 Enterocyte calcium uptake and extrusion: transport mechanisms and endocrine regulation. Recent Research Developments in Membrane Biology. Editor Pandali SG. Transworld research Network. In Press

Conference abstracts

1. Larsson B, Dahlin S, Linde A 1994 Purification of alkaline phosphatase from rat incisor odontoblasts. 77th Annual Meeting of NOF; Göteborg, Sweden
2. Larsson B, Dahlin S Linde A 1995 Partial Purification and Characterization of Alkaline Phosphatase from Rat Incisor Odontoblasts. 5th International

Conference on the Chemistry and Biology of Mineralized Tissues; Kohler Wisconsin, USA

3. Larsson B, Lundgren T, Linde A 1997 Voltage Gated Ca^{2+} Channels in Rat Incisor Odontoblasts. FEBS Advanced Courses Leiden/Amsterdam, The Netherlands
4. Larsson B, Lundgren T Linde A 1997 Voltage-gated Ca^{2+} channels in rat incisor odontoblasts. The 80th Annual Meeting of NOF; Reykjavik, Iceland
5. Larsson B, Gritli-Linde A, Håkanson R, Linde A 1998 Granule content from ECL cells in rat oxyntic mucosa evokes a Ca^{2+} second messenger response in osteoblastic cells. 6th International Conference on the Chemistry and Biology of Mineralized Tissues. Vitell, France
6. Larsson B, Nemere I 2001 Correlation of Growth and Maturation in Roosters with Magnitude of Nongenomic Responses to $1,25(OH)_2D_3$. 23rd Annual Meeting of the American Society for Bone and Mineral Research. Phoenix, Arizona, USA

Commitments and honors

* Referee European Journal of Dental Research (1997).
* Young Investigator award, 6th International Conference on the Chemistry and Biology of Mineralized Tissues, Vittel, France (1998).
* Awarded with the Sweden America Foundation Grant for PhD studies at Utah State University (2000).
* Membership in the Sweden America Foundation (2000).
* Member in the American Society for Bone and Mineral Research (2001).
* Selected for Dean´s List Spring and summer semesters 2001. Biography published in the 24th Annual Edition of the National Dean's List (2000-2001).
* Selected for Dean´s List Fall (2001) and spring (2002) semester. Biography published in the 25th Annual Edition of the National Dean´s List (2001-2002).